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REVIEW

Evaluation of the carcinogenicity of inorganic arsenic

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Abstract

Inorganic arsenic (iAs) at high exposures is a human carcinogen, affecting mainly the urinary bladder, lung and skin. We present an assessment of the mode of action (MOA) of iAs's carcinogenicity based on the United States Environmental Protection Agency/International Programme on Chemical Safety (USEPA/IPCS) framework, focusing primarily on bladder cancer. Evidence is presented for a MOA involving formation of reactive trivalent metabolites interacting with critical cellular sulfhydryl groups, leading to cytotoxicity and regenerative cell proliferation. Metabolism, kinetics, cell transport, and reaction with specific proteins play a critical role in producing the effects at the cellular level, regardless of cell type, whether urothelium, lung epithelium or epidermis. The cytotoxicity induced by iAs results in non-cancer toxicities, and the regenerative cell proliferation enhances development of epithelial cancers. In other tissues, such as vascular endothelium, different toxicities develop, not cancer. Evidence supporting this MOA comes from *in vitro* investigations on animal and human cells, from animal models, and from epidemiological studies. This MOA implies a non-linear, threshold dose-response relationship for both non-cancer and cancer end points. The no effect levels in animal models (approximately 1 ppm of water or diet) and *in vitro* (>0.1 μM trivalent arsenicals) are strikingly consistent. Cancer effects of iAs in humans generally are not observed below exposures of 100–150 ppb in drinking water: below these exposures, human urine concentrations of trivalent metabolites are generally below 0.1 μM, a concentration not associated with bladder cell cytotoxicity in *in vitro* or animal models. Environmental exposures to iAs in most of the United States do not approach this threshold.

Keywords

Arsenic, bladder, cell proliferation, cytotoxicity, epidemiology, lung, skin

History

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Abbreviations: As3mt: Arsenic (+3 oxidation state) methyltransferase; BBN: N-Butyl-N-(4-hydroxybutyl) nitrosamine; BrdU: Bromodeoxyuridine; CDC: Centers for Disease Control and Prevention; DES: Diethylstilbestrol; DMA^{V}: Dimethylarsinic acid; DMA^{III}: Dimethylarsinous acid; DMBA: 9,10-Dimethyl-1,2-benzanthracene; $DMMTA^{V}$: Dimethylmonothioarsinic acid; EPA or US EPA: United States Environmental Protection Agency; HPV: Human papilloma virus; H_2S: Hydrogen sulfide; IC_{50}: Half maximal inhibitory concentration; iAs: Inorganic arsenic; iAs^{III}: Arsenite; iAs^{V}: Arsenate; IARC: International Agency for Research on Cancer; IPCS: International Programme on Chemical Safety; MOA: Mode of action; MMA^{V}: Monomethylarsonic acid; MMA^{III}: Monomethylarsonous acid; NHANES: National Health and Nutrition Examination Survey; NRC: National Research Council; NTP: National Toxicology Program; NOEL: No observed effect level; OPP: Office of Pesticide Programs, EPA; OR: Odds ratio; ODC: Ornithine decarboxylase; PBPK: Physiologically based pharmacokinetic; PML: Promyelocytic leukemia; RR: Relative risk; SAB: Science Advisory Board, EPA; SEM: Scanning electron microscopy; SMR: Standardized mortality ratio; SRRE: Summary relative risk estimates; TPA: 12-*O*-Tetradecanoyl phorbol-13-acetate; TWA: Time weighted average; $TMA^{V}O$: Trimethyl arsine oxide; UUTUC: Upper urinary tract urothelial carcinoma

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Introduction

Inorganic arsenic (iAs) is a known human carcinogen at high exposures, affecting mainly skin, urinary bladder and lungs (International Agency for Research on Cancer (IARC), 2004, 2012; National Research Council (NRC), 1999, 2001). Exposure can be oral or by inhalation. Unlike most chemicals of carcinogenic concern for which the relevance to human carcinogenicity is extrapolated from studies of animal species, information on the carcinogenicity of iAs is available mostly from humans.

In this article, we present the current scientific understanding of the mode of action (MOA) of iAs in the target organs in human and in rodents, as a means for the assessment of risk of carcinogenicity from exposure to inorganic arsenic. We also provide a hypothesis that the cancer and non-cancer toxicities of arsenicals fundamentally have the same MOA, with similar implications for risk assessment.

One of the difficulties in investigating the MOA for iAs carcinogenesis has been the lack of animal models. However, during the past 15 years various animal models have been developed demonstrating the carcinogenicity of arsenicals, including that of rat bladder cancer induced by dimethylarsinate (DMA^V) (Arnold et al., 2006; Cohen et al., 2006a) and transplacental carcinogenesis of iAs in mice (Tokar et al., 2010b, 2011). It is important to point out that monomethylarsonate (MMA^V) has been found to be non-carcinogenic in 2-year bioassays in rats and mice (Arnold et al., 2003).

The MOA by which DMA^V causes urinary bladder carcinogenicity in rats and its relevance to humans was presented to the EPA Science Advisory Board (SAB) in 2005 (US EPA, 2007). This MOA was adopted by the EPA Office of Pesticide Programs (OPP) and has been used as an example for the applicability of the International Programme on Chemical Safety (IPCS) framework to chemicals in general (Dellarco, 2008). Specifically, this MOA involves conversion of DMA^V to the cytotoxic trivalent dimethylarsinous acid (DMA^{III}), which is concentrated in urine, leading to urothelial cytotoxicity, regenerative proliferation, and ultimately to urinary bladder tumors (Cohen et al., 2006a, 2007; Dellarco, 2008). The proliferative lesions known to be associated with carcinogenesis were observed in rats before 10 weeks of oral exposure, but were not detected in mice or in hamsters. This MOA requires generation of sufficient levels of DMA^{III} in the urine to produce cytotoxicity, which clearly implies a non-linear, threshold response.

The evaluation of MOA should consider all of its various aspects, including metabolism, toxicokinetics, cell transport, and cellular responses of the compound and its metabolites. For example, the SAB in its review of DMA^V concluded that differences in toxicokinetics between animal species lead to different responses in those species (US EPA, 2007). This conclusion was substantiated by subsequent research (Clewell et al., 2011; Cohen et al., 2007; El-Masri & Kenyon, 2008; Gentry et al., 2010; Kenyon et al., 2008a; Suzuki et al., 2010; Yokohira et al., 2011).

The MOA of iAs carcinogenicity presented in this article includes a description of all of the aspects listed above, and follows the IPCS mode of action framework (Sonich-Mullin et al., 2001). This framework was originally designed for carcinogens (Meek, 2003), and has evolved over the past decade to include assessment of non-cancer endpoints (Boobis et al., 2006, 2008; Seed et al., 2005). The current framework also covers evaluation of specific modulating factors in potentially susceptible populations, such as the influence of age, nutrition and genetic polymorphisms (Boobis et al., 2008; Seed et al., 2005).

As a general principle, any increased risk for developing cancer is due to either direct damage to DNA or to an increase in cell proliferation, either through direct or indirect mechanisms (Cohen & Arnold, 2011; Cohen & Ellwein, 1990; Moolgavkar & Knudson, 1981). Evidence that has accumulated unequivocally demonstrates that arsenicals do not react with DNA (Nesnow et al., 2002).

Recent studies have demonstrated that iAs follows the same MOA as that of DMA^V in the urinary bladder of rats and mice (Suzuki et al., 2008b, 2010; Yokohira et al., 2011), and evidence is accumulating that the same MOA is relevant also to humans (Xu et al., 2008). Evidence is also accumulating that cytotoxicity and consequent regenerative proliferation is the MOA for iAs-associated skin and lung cancer in humans. Such an MOA implies a non-linear dose-response

relationship, with a threshold. Moreover, epidemiological studies of iAs strongly support a non-linear, threshold phenomenon for tumors in humans (Brown, 2007; Lamm et al., 2006; Lewis et al., 1999; Meliker et al., 2010b; Mink et al., 2008; Steinmaus et al., 2003; Tsuji et al., 2013).

The present article reviews and analyzes the information concerning recent epidemiological investigations, followed by a detailed analysis of the MOA for the carcinogenicity of iAs. The implications for the risk assessment of iAs are then discussed. To provide perspective, this article contains a brief analysis of the MOA for the DMA^V carcinogenesis in rat urinary bladder. This MOA analysis is used as an example of a non-linear dose response involving a threshold for an arsenical, as concluded by the USEPA OPP (US EPA, 2007). Since MMA^V has not been shown to be carcinogenic in any species (animal or human), a cancer MOA analysis for MMA^V is not relevant.

Precautions in evaluating published research

The scientific literature on the underlying mechanisms associated with the toxicity and carcinogenicity of iAs and on the epidemiology of iAs is extensive, with an increasing number of publications over the past few years. However, it has become apparent that several aspects of this vast literature raise important issues as to their usefulness for quantifying, comparing and interpreting the findings, especially regarding relevance to humans and to risk assessment. Some of the issues concern the definition of the arsenic species analyzed, concentrations and doses, types of cells, types of cell cultures, simulation of natural exposures, and extrapolation of *in vitro vs. in vivo* studies. Prior to describing the details of the epidemiology and the MOA analysis for iAs carcinogenicity, the key issues which need to be kept in mind while reviewing the literature are discussed.

Definition of the arsenic species analyzed

There are numerous methodological problems and analytical difficulties involved in detection of trivalent species of organic arsenic (Currier et al., 2011; Hansen et al., 2004; Lu et al., 2003). Analytical methods for arsenite are generally straightforward. However, under certain chromatographic conditions, TMA^VO co-elutes with arsenite giving a falsely increased arsenite concentration, and therefore, in the presence of TMA^VO, specific methods must be used to distinguish between the two compounds (Lu et al., 2003). However, MMA^{III} and DMA^{III} are much more difficult to measure (Currier et al., 2011; Lu et al., 2003). The stability of both is dependent on sample matrix and storage temperature (Currier et al., 2011; Gong et al., 2002). In urine, complete oxidation to the corresponding pentavalent species can occur as rapidly as within one day for DMA^{III} or within up to 5 months for MMA^{III} when storage is at 4 °C or 20 °C (Le et al., 2000). Storage at −80 °C did not improve stability. Currier et al. (2011) showed that MMA^{III} and DMA^{III} were somewhat more stable in a reducing environment found in tissues and cultured cells when storage was at −80 °C. Furthermore, synthesizing valid standards for the trivalent forms is not straightforward, and it has been shown that the assumed trivalent forms can actually be pentavalent forms, leading to erroneous analytical results (Hansen et al., 2004). Continued research is required to provide reliable analytical methods for MMA^{III} and DMA^{III}. Such methods are critical for quantitative comparisons between species, for detection and accurate measurements in human specimens, and for our ultimate understanding of the MOA of the toxicologic effects of the various forms of arsenic.

There are important methodological considerations for measurement of some of the pentavalent arsenic compounds as well. The analytical methods for the majority of pentavalent forms are generally reproducible. Other species are more challenging. Specifically, the analytical methods for TMA^VO are quite limited. In addition to co-eluting with arsenite, it is difficult to synthesize a standard for TMA^VO, and explosions may occur even in the hands of experts (personal communication, Dr. William Cullen). Recently, methods were modified to measure TMA^VO specifically, and a new synthetic process has been developed by Dr. Cullen to make a TMA^VO standard that will be more readily available (personal communication, Dr. William Cullen). When specific methods for TMA^VO have been used, it has rarely been found in human urine specimens; it was identified only when individuals had been exposed to extremely high levels of iAs (Marafante et al., 1987; Xu et al., 2008). This is consistent with the recent finding that DMA^V is a poor substrate for the human enzyme responsible for arsenic methylation, arsenic (+3 oxidation state) methyl transferase (As3mt) (Thomas, 2007). In contrast, DMA^V is a good substrate for rodent As3mt, and TMA^VO is formed readily in these species. In summary, in contrast to rodents, TMA^VO rarely is detected in humans, only at extremely high exposures, and the species differences can be explained by the enzymology.

Definition of concentrations

In vitro studies provide useful mechanistic information only if the concentration of the arsenic compound tested is comparable to the biological concentration in real-life exposure settings. Arsenate is the only pentavalent, oxygen-containing arsenic compound that has any *in vitro* activity at concentrations which occur in such real-life situations (i.e. 1–10 μM). The other pentavalent oxygen-based compounds (MMA^V, DMA^V and TMA^VO) are biologically effective only when the biological concentrations reach millimolar levels (Cohen et al., 2006a; Drobna et al., 2005). In contrast, the effects of trivalent oxygen-containing arsenic compounds occur at concentrations at the micromolar level (<5 μM for arsenite and <1 μM for MMA^{III} and DMA^{III}). *In vitro* studies with concentrations that are higher than biologically realistic concentrations do not provide meaningful mechanistic information and their results cannot be extrapolated to *in vivo* situations in humans (Gentry et al., 2010). For example, Pan et al. (2010) used 400 μM arsenite with yeast mitochondria. High concentrations result in the death of nearly all of the cells, usually within 24–72 hours of exposure (Gentry et al., 2010). Short-term exposure, i.e. 24 hours or less, may not show evidence of overt cell death, but the cytotoxic process would have begun already and thus would distort the biological phenomena being assessed, such as indirect genotoxicity or oxidative damage.

Definition of dose

Dosage is a crucial element for the purpose of measurement of toxicity or carcinogenicity. However, the terms used by researchers are not consistent, making comparison among studies difficult. The doses of iAs in many studies are expressed in units of grams arsenic per kg body weight (g/kg bw) when administration is through the diet or dosing is based on animal body weights, or grams arsenic per liter (g/L) when administration is through the drinking water, referring to the arsenic atoms in the molecule, whether the test compound is arsenite, arsenate or any other arsenic compound (Clewell et al., 2011; Suzuki et al., 2008b). In other publications, the dose or the concentration is expressed in terms of the whole molecule (e.g. grams arsenite per kg) (Cohen et al., 2002; Cui et al., 2006). In others, it is not clear whether the values refer to the arsenic atom or to the entire molecule (e.g. Waalkes et al., 2003). Thus, caution should be taken when quantifying and comparing results of studies. This issue is obviated when information is presented in terms of moles (Cohen et al., 2007).

Cell types used *in vitro*

Most of the information we have on the MOA of arsenic carcinogenesis in humans is based on *in vitro* investigations in cultures of human cells. The limitations of different cell types that were used for extrapolation to *in vivo* conditions must be carefully considered. For example, cells in culture lose many of their normal functions, including differentiation of epithelial cell systems. Cell systems such as urothelium, epidermis and bronchus are more sensitive *in vitro* than *in vivo*, since the fully differentiated epithelium has numerous protective barriers that are not present in the cell culture milieu. Furthermore, frequent passage of cells in culture can result in alterations in cell transport, metabolism, and repair mechanisms (Gentry et al., 2010; Yager et al., 2013).

A number of studies have been performed on malignantly transformed cell lines (Mure et al., 2003), especially in the last decade when research on arsenicals for possible use for cancer chemotherapy has been conducted (Dilda & Hogg, 2007). These studies may not be relevant to the situation in normal or pre-cancerous tissues, since these cell lines already exhibit extensive genetic alterations that greatly modify certain aspects of arsenic metabolism, cell transport, toxic interactions with the cells and repair capabilities.

It is essential to describe carefully the derivation of the cell line that is being used in a study. Furthermore, some cell lines are mischaracterized in publications. For example, A431 cells have been used as representative of skin keratinocytes (Naranmandura et al., 2007), but they are actually derived from a malignancy of the vulva diagnosed as a mucoepidermoid carcinoma with squamous and mucous glandular differentiation (Giard et al., 1973).

Quantitative issues could also arise by use of malignant cell lines, even those derived from tissues known to be targets of iAs carcinogenesis, such as lung carcinoma cells (Bartel et al., 2011). Malignant cells have significant differences compared to their normal counterparts regarding cell transport, metabolism and cellular responses.

Experiments have been performed on mesenchymal cells (e.g. fibroblasts) or on hematopoietic cells (lymphocytes, myelocytic cells). However, studies on fibroblasts derived from organs that are targets for arsenic carcinogenesis are not directly applicable to the epithelia, which are the actual targets for arsenic in the same organ (He et al., 2007). Although the information obtained from these cells can be of value, one has to bear in mind that the tumors arise from epithelia and not from fibroblasts. Although mesenchymal and hematopoietic cells are similar to epithelial cells in the biochemical and molecular responses, there are also striking differences such as mitogenic effects and specific molecular changes (Trouba et al., 1999; Trouba & Germolec, 2004). The results of such studies may serve only as an indicator for a possible effect in the whole organism, but verification is needed to confirm that the effect can also occur in the target epithelial cell. This issue is relevant also to *in vivo* studies, such as the examination of lymphocytes in humans exposed to high levels of arsenic for the ascertainment of possible genotoxic effects.

Recent studies from the Hamner Institute showed that established, immortalized cell lines gave results that are different from findings obtained from primary cell cultures, and therefore can be misleading (Clewell et al., 2011; Gentry et al., 2010; Yager et al., 2013). The use of immortalized cell lines can lead to divergent results when compared to primary cultures, especially if the former has defects in p53. Abnormalities in p53 negate the potential for extrapolation of many *in vitro* observations to *in vivo* situations, particularly in humans (Gentry et al., 2010). This may be especially relevant for investigations related to oxidative stress, DNA repair and *in vitro* transformation, processes in which p53 is involved. In contrast to these cell lines, many of the epithelial tumors that are produced in humans as a result of exposure to arsenic do not have p53 abnormalities (Wu, 2005). In addition, individuals that inherit p53 genetic abnormalities, such as Li-Fraumeni syndrome, do not have an increased incidence of tumors in tissues that are the usual target sites for iAs, such as the urinary bladder, skin and lung (Gonzalez et al., 2009). Thus, p53 may not be critical in the generation of malignancies in humans by exposure to arsenic, so use of cell lines with abnormalities in this gene and/or protein may not accurately represent the process in humans.

Simulation of natural exposures in the laboratory

In an effort to simulate natural situations in studies conducted *in vitro*, researchers have often utilized arsenic concentrations of 1–100 ppb, or μg/L (0.013–1.3 μM) because such concentrations are found in drinking water. However, ingested arsenic reaches the target site in the organism at a concentration that is much lower than in the drinking water. For example, in blood, arsenicals are present at non-detectable levels (detection limits less than 0.01 μM), and in tissues, concentrations of 1 μM are achieved only when the organism is exposed to iA levels greater than 100 ppb iAs in the drinking water (El-Masri & Kenyon, 2008; Kenyon et al., 2008a).

Extrapolation from *in vitro* studies to *in vivo* situations

In vitro investigations can provide useful information, but findings must be verified in the *in vivo* setting (Clewell et al.,

2011; Gentry et al., 2010). Difficulties in interpreting *in vitro* data, as described earlier, and the fact that *in vitro* conditions do not take into account the absorption of the administered arsenical, its metabolism, toxicokinetics and transformation have to be considered when extrapolating to *in vivo* situations in animals and to humans.

In vitro interactions of trivalent arsenicals with proteins do not necessarily occur in the *in vivo* setting. For example, iAs^{III}, MMA^{III} and DMA^{III} react with rat hemoglobin *in vitro*, and yet, when iAs, MMA^{V} or DMA^{V} are administered to the rat, the only form bound to hemoglobin is DMA^{III}, regardless of the administered form (Lu et al., 2007b). It is not clear why iAs^{III} and MMA^{III} do not bind to hemoglobin *in vivo*. However, this example illustrates that while it is possible to investigate various interactions *in vitro*, it is important to identify the proteins that are critical to arsenic binding *in vivo*. Another limiting factor for the analysis of interactions of trivalent arsenicals with tissue proteins *in vitro* is the fact that the cell lines are rarely fully differentiated. Especially in tissues such as urinary bladder urothelium and skin epidermis, cells that are more differentiated may contain specific proteins that interact with arsenic, that are absent in the less diffentiated cell lines.

Extrapolation of *in vitro* studies to *in vivo* situations should be made with caution, as demonstrated also in studies investigating arsenic's therapeutic effects (Dilda & Hogg, 2007; Nicolis et al., 2009). Specifically, arsenic trioxide has shown activity against numerous tumor types *in vitro*, but has been clinically useful only in the treatment of promyelocytic leukemia (PML) (Lu et al., 2007a; Zhang et al., 2010). All these issues must be kept in mind when reviewing the literature, comparing the findings of the various studies, interpreting the results and making conclusions with regard to the metabolism, the MOA and the possible risks of arsenic.

Measurement of exposure in epidemiological studies

Accurate exposure measurement is critical for the reliable assessment of health risks in epidemiological studies in general, and in the assessment of iAs, specifically.

Errors in exposure estimates, while common in epidemiological studies, seldom receive adequate consideration, particularly with respect to their impact on dose-response relationships. Many investigators assume that errors in exposure estimation always result in non-differential misclassification in which some exposed individuals are classified as non-exposed and some non-exposed are classified as exposed such that any exposure misclassification always biases results toward the null (i.e. the resulting dose-response relationships are weaker than it might be if exposure were more accurately characterized). However, several researchers have demonstrated that this assumption is incorrect, and in some cases, errors in exposure estimation lead to an artificially increased dose-response relationship (Crump, 2005; Jurek et al., 2008).

Sources of error in exposure estimates in iAs epidemiology studies derive from failure to specifically measure inorganic arsenic, or from overlooking certain sources of exposure.

The most common source of iAs exposure is from water (drinking water, beverages, foods made with water). Additional sources are consumption of certain foods which are high in iAs, and of soil. In occupational settings, inhalation of iAs in air can be an important route of exposure.

Most studies calculate exposure to the combined trivalent and pentavalent forms, and express exposure in terms of total iAs. This is appropriate, because most of the ingested pentavalent inorganic form of arsenic (arsenate) is rapidly and nearly completely converted in the body to the trivalent inorganic form (arsenite). Therefore, the form of inorganic arsenic in water is usually not as critical as the form of arsenic in foods containing non-toxic organic forms of arsenic, such as arsenobetaine and arsenosugars that can result in misleading exposure assessments if total arsenic is measured instead of inorganic arsenic.

However, there are other important sources of error in evaluating exposure to iAs in drinking water. For example, studies frequently use a single or only a few measurements to estimate iAs in drinking water. Because concentrations of iAs can vary over time (as was demonstrated in dramatic changes in drinking water associated in water in Antofagasta, Chile where average As drinking water concentrations increased more than 600 μg/L in the span of 1 year when the key drinking water resource changed (Smith et al., 1998), failure to consider such temporal changes, particularly with studies involving exposures over years, can yield both over- and underestimates of exposure.

It is also important to consider possible sources of error with respect to drinking water consumption patterns. For example, individuals consume drinking water in multiple locations (home, school, work) and failure to account for the amount consumed in these different locations and the associated iAs concentrations can lead to errors in exposure estimates, particularly for relatively mobile populations as in the US. (See Meliker et al. (2010a) for a further discussion on this topic.) Self-reported drinking water histories are another source of uncertain exposure esitimates. Efforts to provide more accurate estimates of drinking water exposures are underway. Specifically, several researchers have used publicly available databases on residential history to obtain more accurate information on at-home drinking water exposures (Jacquez et al., 2011).

Urine concentrations of iAs and its metabolites have been used as a biomonitor of exposure (ATSDR, 2012a). While iAs in urine can be a useful metric of exposure in that it reflects iAs intake from all sources, it reflects relatively recent exposure and thus must be used cautiously when evaluating long-term exposure. In addition, urinary concentrations can be influenced by other variables, such as urine volume and density. To adjust for variations due to urine density, some investigators express urinary concentration per unit of creatinine, since creatinine varies according to urine volume (e.g. Valenzuela et al., 2005). Thus, such adjustments are believed to account for differences in urine volume across individuals. However, studies suggest that creatinine might vary with the underlying disease, such as diabetes, and nutritional status in individuals, complicating its use in "normalizing" concentrations of urine arsenicals (Gamble & Hall, 2012).

For assessments of longer-term iAs exposure, levels in hair or nails have also been used. Hair poses a problem due to contamination from water used in washing and other sources. Nails can be useful in evaluating longer-term iAs exposure (on the order of months) and can be collected more readily than blood or urine (ATSDR, 2012b). However, as with urine, nails still reflect a relatively short-term exposure, especially in the context of needing to understand exposure duration over years for carcinogenicity assessment. Also, it is difficult to relate toenail iAs to a specific dose, making it challenging to use such findings with respect to risk assessment.

In summary, many epidemiological studies of iAs, underestimate exposures, which result in overestimates of the risk from iAs (Meliker, et al., 2010a, 2010).

Epidemiology of inorganic arsenic and cancer

The following sections review the epidemiological research of the association between human exposure to iAs and cancers of the urinary bladder, lung and skin. Most of the studies that were reviewed refer to exposure to "arsenic" without specifying the molecular form (i.e. inorganic or organic).

Urinary bladder cancer

The first reports of epidemiological studies which investigated the association between exposure to iAs in drinking water and urinary bladder urothelial carcinomas were published in the 1980s. Those publications reported a series of ecological investigations that were conducted in southwest Taiwan in the 1950s (Chen et al., 1985, 1986, 1988a). While a dose-response relationship was demonstrated in those studies, the ecological study design and the exposures to high concentrations of iAs make these studies difficult to use for the assessment of the risks of low exposures.

Since these first publications, additional studies using ecological designs have continued to demonstrate a relationship between inorganic arsenic ingestion and bladder cancer. While ecological studies are less useful for establishing reliable dose-response relationships and understanding low-dose extrapolations, cohort and case control designs have better informed the quantitative relationship between human exposure to high concentrations of iAs in drinking water and bladder cancer. These studies (ecological, case-control and cohort) have been evaluated in the peer-reviewed literature (Begum et al., 2012; Petito-Boyce et al., 2008; Schoen et al., 2004), as well as by government agencies and other organizations (IARC, 2004, 2012; NRC, 1999, 2001; US EPA, 2010). In this section, we briefly summarize the literature through 2004, as reviewed by Schoen et al. (2004), and provide details on more recent studies. Based on literature through 2004, Schoen et al. (2004) concluded that an increase in bladder cancer is observed only in humans who were exposed to iAs in drinking water at high concentrations, usually several hundred μg/L. For example, a cohort study by Chiou et al. (2001) found no statistically significant increase in bladder cancer in a northwest Taiwanese population that consumed drinking water containing iAs at concentrations up to 100 μg/L. A significant increase was observed only in the group consuming water in which the average concentration of iAs was above 100 μg/L. This group included individuals exposed to water with iAs levels greater than 600 μg/L. A case-control study conducted in Argentina by Bates et al. (2004) resulted in similar findings: no statistically significant increase in bladder cancer was observed in groups of individuals consuming drinking water with average iAs concentrations of 0–50, 51–100, 101–200, and even >200 μg/L.

Overall, studies conducted in US states such as Utah, Nevada and California, have not found an association between exposure to iAs and an increase in bladder cancer. These included case-control studies by Bates et al. (1995) and Steinmaus et al. (2003), a cohort analysis by Lewis et al. (1999) and an ecological study by Lamm et al. (2004). In these studies the exposure was to water containing iAs at concentrations up to a mean of almost 200 μg/L. Within some studies, the maximum levels of iAs were up to 1000 μg/L. When the analysis was restricted to smokers, there were some instances of a statistically significant increase in bladder cancer, but the results were inconsistent across different latency periods and metrics of exposure (Bates et al., 1995; Steinmaus et al., 2003).

Studies published after 2004 confirm that the association between exposure to iAs in drinking water and bladder cancer is found only after exposure to iAs concentrations that are greater than 100 μg/L. For example, Yang et al. (2005) evaluated mortality from bladder cancer over a follow-up period of 30 years in a southwest Taiwanese population that had been exposed to iAs in drinking water at concentrations up to 780 μg/L, before and after installation of water treatment systems. The authors demonstrated that installation of water treatment systems resulted in a decline in mortality from bladder cancer in the exposed population. A similar decline in bladder cancer due to installation of drinking water treatment systems was observed in a Chilean population that had been exposed to iAs in water at concentrations up to 870 μg/L, for several decades (Marshall et al., 2007). Recently, however, Steinmaus et al. (2013) reported that the incidence of bladder cancer although decreased, remains moderately elevated in the Chilean residents that had been exposed to the highest levels of iAs (average well water arsenic >91–335 or >335 μg/L), indicating that the risk of iAs-induced bladder cancer may persist, similar to bladder cancer risk associated with cigarette smoking (Cohen et al., 2000). In a lower exposure study conducted in the US, Meliker et al. (2010b) found no increase in bladder cancer incidence in a population consuming drinking water containing iAs at concentrations of 10–100 μg/L. This finding was consistent with results from an earlier ecological study by the same research group, which showed no increase in bladder cancer mortality in Michigan (Meliker et al., 2007).

Further analyses of the southwest Taiwanese cohort have been conducted by several researchers. For example, Lamm et al. (2006) reanalyzed the original dataset (NRC, 1999; Wu et al., 1989), and demonstrated that geographically related risk factors for bladder and lung cancer, other than iAs, confounded the results of the previous analyses. The analysis by Lamm et al. (2006) demonstrated that a significant association between exposure to iAs and incidence of cancer (including bladder cancer) occured only in three of the six townships where the study had been conducted. Furthermore,

the dose-response relationship in these three townships was found to be non-linear, with a threshold for increased risk at approximately 150 μg/L. Brown (2007) demonstrated a similar dose-response relationship in the same population using a different method of analysis: villages with multiple wells in which the difference in iAs concentrations in drinking water between two wells was more than 25 μg/L were excluded. After making this adjustment, the dose-response relationship between exposure to drinking water with iAs concentrations less than 100 μg/L and cancer, showed a "flat or downward" trend. Similar to the conclusions of the analysis by Lamm et al. (2006), a high cancer background rate was apparent in these villages (Brown, 2007), unrelated to exposure to iAs.

To better understand low-dose iAs effects, Lamm et al. (2013) further analyzed data from 18 of the low-dose villages (<150 μg/L in well water) in the Taiwanese cohort using Poisson regression methodology. The authors performed analyses of the villages based on three different exposure metrics: a median of <150 μg/L, a mean of <150 μg/L, or a maximum of 150 μg/L iAs. When the entire southwest Taiwanese population was included in a comparison to the population in the arseniasis endemic area, there was a small positive slope. However, excluding an external comparison population resulted in a negative dose-response slope up to 150 μg/L iAs (using all metrics, mean, median and maximum).

To address claims that the lack of significantly increased bladder cancer risk was due to lack of study power by the low-level exposure studies, Mink et al. (2008) conducted a meta-analysis of data from eight studies of low levels of iAs which were selected with strict inclusion criteria of study design, appropriate biomarkers, comparison populations, and nutritionally sufficient populations. The authors created models to evaluate all study participants, and conducted several sensitivity analyses with control for smoking. The studies evaluated were:

- A case control study of bladder cancer in Utah (Bates et al., 1995)
- A cohort study of bladder cancer in Utah (Lewis et al., 1999)
- A case control study of bladder cancer in Finland (Kurttio et al., 1999)
- A cohort study of urothelial cell carcinoma in residents from an arseniasis-endemic area in northeastern Taiwan (Chiou et al., 2001)
- A case-control study of bladder cancer in the western United States (Steinmaus et al., 2003)
- A case-control study of bladder cancer in Argentina (Bates et al., 2004)
- A case control study of urothelial cell carcinoma of the bladder in New Hampshire (iAs in toenails as a measurement of exposure) (Karagas et al., 2004).
- A cohort study in Finland of bladder cancer (iAs in toenails as a measurement of exposure) (Michaud et al., 2004).

In the first six of the eight studies, the exposure measurements were of iAs concentrations in drinking water; the other two used arsenic in toenails as the measurement for exposure.

The meta-analysis resulted in no significant associations between exposures to low levels of iAs in drinking water (typically <100–200 μg/L) and bladder cancer [for never-smokers summary relative risk estimates (SRRE = 95% CI, p-heterogeneity) = 0.81, 95% CI: 0.60–1.08, 0.937; for ever-smokers SRRE = 1.24, 95% CI: 0.99–1.56, 0.032; and for combined never-smokers and ever-smokers, SRRE = 1.11, 95% CI: 0.95–1.30, 0.207]. To increase precision and statistical power, the meta-analysis was updated in 2013 with two additional studies that were published in 2010 (Chen et al., 2010b; Meliker et al., 2010b). Even with the improved precision and increased statistical power, the conclusions of the revised meta-analysis remained the same [SRREs (95% CI, p-heterogeneity) = 1.07 (95% CI: 0.95–1.21, 0.543) for all individuals, RR = 0.85 (95% CI: 0.66–1.08, 0.915) for never smokers and 1.18 (95% CI: 0.97–1.44, 0.034) for ever-smokers] (Tsuji et al., 2013). Additionally, this analysis demonstrated that risks predicted by the NRC in the 2001 assessment for low exposures (equivalent to an iAs slope factor about 23 mg/kg-d^{-1}) were incompatible with the results of the meta-analysis.

The multiple epidemiologic studies support the conclusion that low exposure to iAs does not increase the risk of bladder cancer, and the most recent meta-analysis (Tsuji et al., 2013) indicates that the incidences predicted by a linear extrapolation from incidences at high exposures are not compatible with empirical findings. As described by Mink et al. (2008), it is unlikely that exposure misclassification/regression to the null explains this lack of a significant positive relationship, casting doubt on the linear model used by the NRC (2001).

Recently, Begum et al. (2012) conducted a comprehensive meta-analysis of data of iAs and internal cancers through 2010. Inclusion criteria were the following: (1) internal cancer as the endpoint or health outcome; (2) long-term exposure at low levels of InAs in drinking water or evidence of such exposure; (3) well-defined epidemiological study designs; and (4) relative risk estimates with measures of variability and availability of covariate information or stratified analysis. Ten studies of bladder cancer met the inclusion criteria, including Bates et al. (1995), Chiou et al. (2001), Steinmaus et al. (2003), Bates et al. (2004), Karagas et al. (2004), Kurttio et al., 1999, and Michaud et al. (2004), which were evaluated in the Mink et al. (2008) meta-analysis. In addition to these studies, Begum et al. (2012) also included in their analysis:

- A case control study of bladder cancer in Michigan (Meliker et al., 2010b).
- A case-case study of bladder tumors and the prevalence of p53 mutations and protein expression in South America (Moore et al., 2003).
- A "follow-up" cohort study of patients with black foot disease and bladder cancer in Taiwan (Chiou et al., 1995).

All studies except Moore et al. (2003) examined populations subject to iAs exposure levels less than 100 μg/L. Begum et al. (2012) reported that the plotted dose-response relationships did not show a consistent pattern across studies. A dose-response model used by the authors yielded a combined slope estimate of 0.002387; thus, the authors concluded that there was minimal risk of bladder cancer at low exposure levels. Based on bladder cancer prevalence data from the National Cancer Institute, the authors estimated that a maximum contamination level of 10 μg/L would be

associated with about 2.91 additional bladder cancer cases per 100 000 people. It should be noted that this estimate is based on a linear dose-response model for iAs and bladder cancer with no threshold. Use of a model which is more biologically-supported (as discussed below) indicates that no bladder cancer cases occurred at 10 μg/L.

Chen et al. (2010a) reported a cohort study from Taiwan that examined the association between ingestion of well water containing different concentrations of iAs and the incidence of urinary tract carcinomas. The researchers reported that the association was statistically significant only at iAs concentrations greater than 100 μg/L [Relative risk (RR) for arsenic concentrations of 100–299.9 μg/L = 4.13 (95% CI: 1.32–12.9); RR for arsenic concentrations above 300 μg/L = 7.80 (95% CI: 2.64–23.1)]. There is uncertainty in the exposure analysis since the exposure in this study was based on current iAs levels, and was not measureable for 15% of the study participants. However, the long follow-up, large sample size and adjustment for important confounders (such as cigarette smoking and alcohol consumption), increase the reliability of the study results.

Meliker et al. (2010b) reported a case-control study that was conducted in southeastern Michigan, where no association was found between exposure to low levels of iAs (less than 100 μg/L) and bladder cancer in ever-smokers. The authors reported an association between time weighted average (TWA) of iAs exposure and risk of bladder cancer when using continuous measures in never smokers, but no association was observed for continuous measures in all subjects or in smokers or for any of the categorical analyses for all subjects, ever, or never smokers. In fact, the direction of the odds ratio for the middle dose category in never smokers was less than 1.0. The authors cautioned against inferring an increased risk for non-smokers for several reasons, including the limited number of never smokers and the inconsistency with the results of other studies.

In 2011, Pou et al. (2011) published an ecological study of a population in Argentina in which they found statistically significant increases in mortality due to bladder cancer in females and males exposed to high levels of iAs (320–1800 μg/L), and in males exposed to intermediate (40–320 μg/L), and low (0–40 μg/L) iAs concentrations. Several factors make the results of this study highly uncertain and questionable from a quantitative perspective. For example, bladder cancer rates for highly exposed men were compared with rates for low exposed women (rather than men), in spite of differences between Argentinean men and women in bladder cancer rates (age-standardized incidence rate per 100 000 people: males, 15; females, <3). In addition, the authors used lung cancer mortality rates as a surrogate variable to adjust for smoking status in the Argentine population, although these rates may overestimate or underestimate actual smoking rates. The study found, for both men and women, a reduction in overall bladder cancer mortality rates over time (1986–2006), which the authors attribute to a reduction in exposure to arsenic due to improvements in water quality. Other potential causes for the decline, such as improved medical care, early detection, decreased smoking incidence, and various lifestyle improvements, were not evaluated (Pou et al., 2011). Because of all these factors the significant increases in risk for men may be unrelated to iAs exposure.

In a recent re-analysis of the Chilean population exposed prenatally and/or during childhood to water contaminated with iAs up to 870 μg/L ($n = \sim$60 000), Smith et al. (2012) reported an increased risk of mortality from bladder cancer for both sexes (Standardized Mortality Ratio (SMR) = 18.1, 95% CI: 11.3–27.4). When the analysis was restricted to individuals with probable *in utero* and childhood exposure, risks were significantly higher for interaction between the two birth periods, adjusted for gender (SMR = 43, 95% CI: 8.9–126 for females and SMR = 65.7, 95% CI: 24.1–143 for males, $p = 0.01$).

Fernandez et al. (2012) evaluated cancer incidence and mortality in residents of the same area of Chile during 1983–2009 (up to 20 years after the iAs levels in the drinking water were reduced). The authors reported that hospital discharge rates due to bladder cancer were significantly higher in this region compared to the rest of the country (RR = 3.6, 95% CI: 3.0–4.7), and mortality from bladder cancer was also significantly higher (RR = 5.3, 95% CI: 4.8–5.8 for men, and RR = 7.8, 95% CI: 7.0–8.7, for women).

Wang et al. (2012) found an increased risk of bladder or upper urinary tract urothelial carcinoma (UUTUC) only in non-smoking Taiwanese exposed to iAs in drinking water at concentrations of ≥350 μg/L for 10 years or more. However, when the authors considered smoking status, ever-smokers in the low exposure group (<350 μg/L) had an increased risk for bladder cancer [Odds Ratio (OR) = 2.7, 95% CI: 1.9–3.6] and UUTUC (OR = 2.2, 95% CI: 1.4–3.4). Ever-smokers exposed to high levels of iAs had the greatest risk for both bladder cancer (OR = 5.7, 95% CI: 3.1–10.3, p for trend <0.001) and UUTUC (OR = 6.4, 95% CI: 3.1–13.3, p for trend <0.001). Furthermore, smokers with one or more risk genotypes of the *VEGF* gene had even higher risk (OR = 6.6, 95% CI: 3.1–13.9 for bladder cancer; OR = 9.9, 95% CI: 4.0–24.5 for UUTUC). The results of Wang et al. (2012) are consistent with the relationships observed in the meta-analysis performed by Mink et al. (2008).

Overall, epidemiological studies continue to support a threshold for bladder cancer associated with exposure to iAs in drinking water, although the exact threshold varies among studies. Some studies, including the meta-analyses, reported a threshold of around 100–150 μg/L (Chen et al., 2010a; Mink et al., 2008; Tsuji et al., 2013), while others found an association only above 300 μg/L (Marshall et al., 2007; Wang et al., 2012; Yang et al., 2005). Results from different geographical areas indicate a threshold for association between iAs exposure and cancer of the bladder at concentrations of 100 μg/L or higher.

Lung cancer

Associations between lung cancer and ingestion of high doses of iAs in drinking water have been observed in Taiwan (Chen et al., 1988a,b, 2004a; Guo, 2004) and in South America (Marshall et al., 2007; Smith et al., 2006), but these studies are not sufficient to evaluate the potential of iAs to cause lung cancer at low exposures. For example, Guo (2004) examined the relationship between lung cancer mortality and iAs

concentrations in drinking water, based on death certificates in Taiwanese townships, and found a significant increase in lung cancer mortality in men and women exposed to drinking water with iAs levels that are greater than 640 μg/L. Smith et al. (2006) found a significant increase in lung cancer mortality in a region of Chile where iAs concentrations in water ranged from 90 μg/L to nearly 1000 μg/L for a period of approximately 13 years [Standardized mortality ratio (SMR) = 7.0, 95% CI: 5.4–8.9, $p < 0.001$], for a cohort exposed to iAs during their childhood, all born just before the peak exposure period. A similar increase in risk was found for a cohort exposed to the same concentrations prenatally and during their childhood (SMR = 6.1, 95% CI: 3.5–9.9).

Several studies of lung cancer and iAs ingestion in populations outside the US have recently been published. For example, Mostafa et al. (2008) published a case-control study of a population in Bangladesh, and Chen et al. (2010b) published a cohort study from Taiwan. Lamm et al. (2006, 2013) have also published several re-evalutions of the Taiwanese data set that EPA has previously used to develop the iAs cancer slope factor. All these studies support a threshold for iAs carcinogenicity of the lung at approximately >100 μg/L.

Mostafa et al. (2008) evaluated iAs concentrations in four ranges: 0–10, 11–50, 51–100, and 101–400 μg/L. Results of data stratified by smoking showed no significant associations at any exposure levels for male non-smokers, but a statistically significant association between the highest exposure level, i.e. iAs concentration of 101–400 μg/L, and increased incidence of lung cancer in male ever-smokers.

Chen et al. (2010b) found no significant association between ingestion of well water containing iAs at levels of 300 μg/L or less and lung cancer among never-smokers. The odds ratio (OR) was 2.25 for iAs concentrations in drinking water that are greater than 300 μg/L (95% CI: 1.43–3.55). The threshold was found to be modified by smoking. In a follow-up analysis with exposure ranges of <10 μg/L, 10–99.9 μg/L and >100 μg/L, there was no statistically significant increase of lung cancer in never-smokers. In smokers reporting a smoking history of fewer than 25 pack-years, a statistically significant increase in risk of lung cancer (OR = 5.30, 95% CI: 2.19–12.8) was found with ingestion of well water containing iAs at concentrations of more than 100 μg/L. For those individuals reporting a smoking history of 25 pack-years or more, a statistically significant increased risk of lung cancer was observed in all of the iAs exposure ranges compared to never-smokers. The relative contribution of iAs *vs* smoking could not be assessed from the available data.

Similar to the bladder cancer anlaysis, using Poisson regression analysis for low-exposure areas of Taiwan (<150 μg/L), Lamm et al. (2013) reported a positive upward trend in the dose-response relationship between iAs and lung cancer when the rest of southwestern Taiwan was included as an additional ''low-dose'' area. However, the authors reported a significant downward trend in lung cancer incidence when the 18 low-exposure areas were analyzed without a comparison population.

In a recent meta-analysis, Begum et al. (2012) evaluated the dose-response relationship between iAs and lung cancer in five studies:

- A case-control study of iAs in drinking water and lung cancer in New England (Heck et al., 2009)
- A case-control study of lung cancer in Northern Chile (Smith et al., 2009).
- Three follow-up cohort studies of an iAs-endemic area in southwestern and/or northeastern Taiwan (Chen et al., 2004a, 2010b; Chiou et al., 1995).

Similar to the results for bladder cancer, the fixed-effects model of the studies yielded a modest combined slope estimate of 0.01082. An even lower slope factor of 0.004581 was observed when only studies conducted since 2000 were included. Based on the four studies that were conducted after 2000, the authors estimated that a maximum contamination level of 10 μg/L iAs would be associated with about 4.5 additional lung cancer cases per 100 000 people (absolute risk) in the US. As noted earlier, however, this calculation assumes a non-threshold dose-response relationship, which is not consistent with what is known of the biologic effects of iAs.

Studies in the US involving median exposures to iAs (i.e. up to approximately 150 μg/L, which are below exposure levels showing an increased incidence of lung cancer outside the US) have not found a statistically significant increase of lung cancer in association with iAs ingestion, overall (Lewis et al., 1999).

Skin cancer

Skin cancer was the first documented carcinogenic effect from chronic exposure to iAs, and has been studied extensively. It was first identified in individuals treated with solutions containing high concentrations of iAs such as Fowler's, Gay's, or others, for therapeutic purposes (Cullen, 2008; Hughes et al., 2011). Paul Ehrlich received a Nobel Prize in 1908 for the development of an iAs-containing antibiotic that could be used for the treatment of syphilis. The treatment was limited due to skin changes produced by the arsenical, identical to those caused by exposures to high levels of iAs in the drinking water (Cullen, 2008).

The association between iAs in drinking water and skin cancer has been observed in a number of studies conducted in Taiwan (Chen et al., 1985, 1988a,b; Chen & Wang, 1990; Guo et al., 2001; Tsai et al., 1999; Tseng, 1977; Tseng et al., 1968; Wu et al., 1989), as well as in Argentina, Australia, Chile and Mongolia (Hopenhayn-Rich et al., 1998; Hinwood et al., 1999; Smith et al., 1998; Tucker et al., 2001). Overall, these studies indicate increases in skin cancer in populations with high exposure to iAs in drinking water (300–1000 μg/L). For example, Guo et al. (2001) examined the relationship between iAs and skin cancer in 243 Taiwanese townships in a study with ecological design. After adjustment for urbanization and age, they found a significant increase ($p < 0.01$) of basal cell carcinoma in populations exposed to drinking water with iAs levels greater than 640 μg/L. Squamous cell carcinomas increased significantly in those exposed to iAs levels of 170–320 μg/L, and surprisingly decreased significantly at 330–640 μg/L. In addition to high doses, it appears that skin cancers also increase after long periods of exposure, generally decades (NRC, 1999, 2001).

Using a case-control study design, Chen et al. (2003a) found an elevated risk for skin cancer (OR = 2.99, 95%

CI: 1.30–6.87) in the population of southwest Taiwan with the highest cumulative iAs exposure (>15 000 μg/L-year), compared to the population with the lowest cumulative iAs exposure (0–2000 μg/L-year). In an ecological study in Inner Mongolia, where well water was reported to contain iAs levels of up to 2000 μg/L, Lamm et al. (2007) identified skin cancer cases, including non-melanomas, only among the population exposed to iAs concentrations greater than 150 μg/L. Additional modeling (hockey-stick model), in this same publication showed a statistically significant ($p < 0.05$) threshold for skin cancer at 122 μg/L (95% CI: 88–137).

Baastrup et al. (2008) conducted a cohort study with 57 000 people to evaluate a possible association between exposure to iAs and skin cancer in a Danish population exposed to drinking water containing iAs levels at a concentration range of 0.05–25.3 μg/L (mean = 1.2 μg/L). The researchers found no increased risk for skin melanoma or non-melanoma cancers from exposure to iAs in drinking water, whether calculated as cumulative or as time-weighted average exposure.

In the US, no consistent statistically significant association has been found between iAs ingestion and increased incidence of skin cancer (basal cell carcinoma and squamous cell carcinoma, although usually not distinguished in most studies). For example, Karagas et al. (2001) used a case-control study to examine the relationship between iAs concentrations in toenail as a measure of exposure, and various skin neoplasms among skin cancer patients in New Hampshire. While the study results suggested an association between iAs content in toenail and skin cancer, the relationship was not statistically significant, even for the highest exposure category. Beane-Freeman et al. (2004) also used toenail iAs concentration as a measure of exposure to iAs to study the association with cutaneous melanoma in a case-control study of a population in Iowa, and found a significant linear trend ($p_{trend} = 0.001$). Arsenic is not known to be a risk factor for melanoma; as described above, the types of skin cancer with an established relationship to arsenic exposure are basal cell carcinoma and squamous cell carcinoma (Guo et al., 2001; IARC, 2012). A relationship between iAs ingestion and higher prevalence of skin cancer was also reported by Knobeloch et al. (2006) in a cross-sectional study in rural Wisconsin for a population that had consumed drinking water with iAs levels greater than 1.0 μg/L for at least 10 years, compared to those whose drinking water contained less than 1.0 μg/L (for arsenic levels of 1.0–9.9 ppb: OR = 1.81, 95% CI: 1.10–3.14). In this study, the drinking water contained iAs at levels ranging from <1.0 to 3100 μg/L (with a median of 2 μg/L). The results of this study should be viewed with caution because of several limitations and biases: families were self-selected for arsenic analysis; no distinction was made between melanoma and non-melanoma skin cancer; cancer status was self-reported, introducing inaccuracies in date of diagnosis or skin cancer types; and a causal relationship cannot be concluded from a cross-sectional study (i.e. timing/amount of exposure in relation to measured disease is uncertain). Additionally, because the exposed population ingested drinking water containing iAs concentrations ranging from 1.0 to 3100 μg/L, no reliable iAs estimates can be inferred regarding the exposures of the individuals with skin cancer, greatly adding to study uncertainty. A similar association has not been reported in any reliable study.

Meliker et al. (2007) found no consistent associations for skin melanoma or other skin cancer types (including basal cell carcinoma and squamous cell carcinoma) in males or females, in an ecological study across six counties in southeastern Michigan. SMRs for other skin cancer types were statistically significantly high (SMR = 1.61, 99% CI: 1.05–2.36) only in males and only in one (Genesee) of the six counties. This county varied from the other counties of the study also in the percentage of rural population (21% in Genesee compared to 80% in the other counties), so the differences in mortality rates may reflect other differences between urban and rural populations.

In a case-control study, Leonardi et al. (2012) evaluated associations between iAs in drinking water and skin cancer (basal cell carcinoma) in residents of Hungary, Romania and Slovakia. In general, ORs increased with increasing exposure to iAs (trend test p values were 0.001 for each exposure measure). Statistically significant increases in risk occurred only in the highest average lifetime exposure group (19.54–167.29 μg/L). However, due to concerns that iAs in the diet was biasing the results, the authors conducted an additional analysis that included participants whose total urinary iAs concentrations were >2.5 μg/L (under the assumption that iAs in the diet would not be a large contributor to iAs exposure in that group). The analysis was further truncated to include only those individuals with average lifetime concentration of 40.7 μg/L (90^{th} percentile exposure) or less. Using these criteria, the authors identified a statistically significant OR for basal cell carcinoma (OR = 1.41, 95% CI: 1.14–1.76).

It should be noted that the Leonardi et al. (2012) study is the only one which demonstrates an increased incidence of basal cell carcinoma at levels less than 50 μg/L iAs in drinking water, and thus, is inconsistent with many other studies showing increased risk at much higher concentrations. There are several concerns with this study, such as possible bias associated with rural *vs* urban differences (e.g. iAs drinking water concentrations) and an unexplained increase in OR when the analysis excluded individuals with the most exposure (>90^{th} percentile). Thus, the study results should be viewed with caution.

In conclusion, overall, well-designed studies published through 2013 generally find a reliable relationship between ingestion of iAs and skin cancer only at drinking water concentrations exceeding 100 μg/L; similar to findings for bladder and lung. This epidemiological evidence strongly indicates a threshold for iAs-related cancer, including urinary bladder, lung and skin. To substantiate such a conclusion an understanding of the mode of action is required. The analysis of the MOA for iAs-induced cancer presented in the following pages strongly supports a non-linear dose-response relationship with a threshold, which is consistent with the epidemiologic findings of the studies described above.

Arsenic metabolism

To understand the carcinogenicity of arsenic, one must first understand the basic chemistry and metabolism of the various

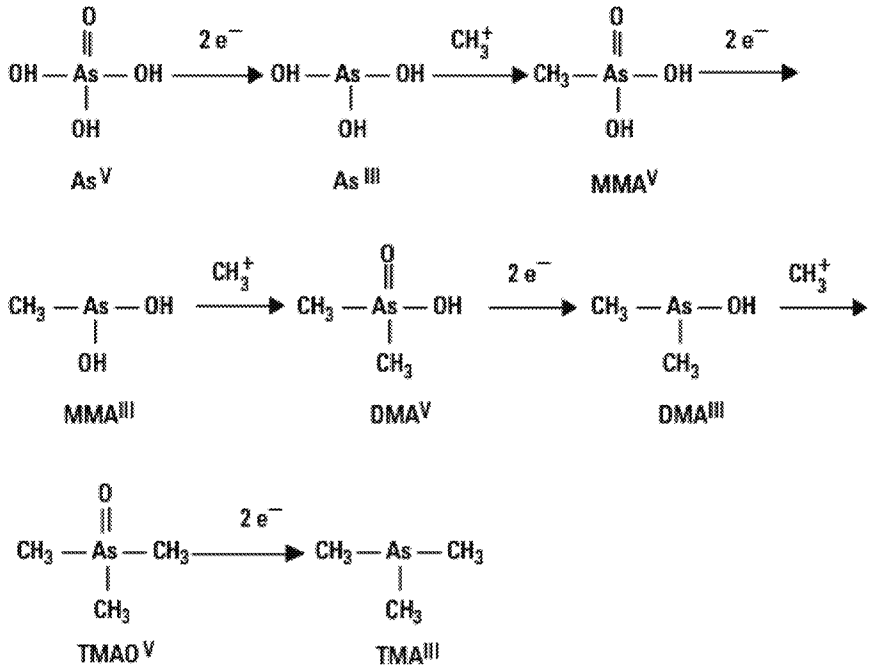

Figure 1. Metabolism of inorganic arsenic through a series of reductions and oxidative methylations (from Le et al., 2000), as originally proposed by Challenger (1951). Used with permission.

forms of arsenic. The metabolism of iAs (Figure 1) involves a series of reductions from the pentavalent oxidative state to the trivalent state, alternating with oxidative methylations, resulting in the sequential addition of methyl groups to form pentavalent MMA^V, DMA^V and TMA^VO (Cohen et al., 2006a; El-Masri & Kenyon, 2008; Kenyon et al., 2008a; Thomas, 2007). The trivalent forms of arsenic, especially organic trivalent arsenicals, are highly reactive and cytotoxic, with activity at low micromolar concentrations (Cohen et al., 2006a). The organic pentavalent forms of arsenic are mostly much less reactive, with activity generally at millimolar concentrations. In large part, this is due to the fact that, unlike the trivalent forms, the pentavalent forms of arsenic are not easily transported across the cell membrane (Drobna et al., 2010; Hughes et al., 2008; Thomas, 2007). Furthermore, the reactivity of the pentavalent forms with sulfhydryl groups is considerably lower than that of the corresponding trivalent forms (Cullen, 2008; Kitchin & Wallace, 2008; Le et al., 2000).

In the environment, arsenic occurs mainly as iAs, existing in several forms, primarily in the pentavalent form as arsenate (iAs^V) and in the trivalent form, as arsenite (iAs^{III}) (NRC, 1999, 2001). Exposure occurs primarily *via* drinking water, but dietary exposures can also be substantial. iAs is metabolized by most organisms to organic arsenicals, mainly to the monomethyl and dimethyl forms, but trimethyl forms, such as trimethyl arsine oxide (TMA^VO), also occur (Hughes et al., 2008; Thomas, 2007). Arsenobetaine, arsenocholine, several arsenosugars (Andrewes et al., 2004; Francesconi et al., 2002) and arsenolipids (Fukuda et al., 2011; Taleshi et al., 2010) occur in sea organisms and in some food substances. These generally are not activated metabolically in mammalian species, including humans (Navas-Acien et al., 2011). However, recent limited data suggest that some of the arsenosugars may be converted, to some extent, to DMA^V, which is excreted in the urine (Borak & Hosgood, 2007; Heinrich-Ramm et al., 2002; Heitland & Koster, 2008; Le et al., 1999, 2004; Molin et al., 2012; Raml et al., 2009; Wei et al., 2003). Evidence is also accumulating that food other than seafood (e.g. rice) may also contain and provide significant exposure to arsenicals, including iAs and small amounts of DMA (Gilbert-Diamond et al., 2011; Jackson et al., 2012; Xue et al., 2010). Exposure may also occur in occupational settings (Farmer & Johnson, 1990), as well as from traditional medicines as realgar and cinnabar (Liu et al., 2011; Lu et al., 2011) and in western medicine as arsenic trioxide (Dilda & Hogg, 2007; Zhang et al., 2010) or darinasparsin (investigational use only) (Tian et al., 2012), although, occupational and medicinal exposures account for a very small proportion of human exposures.

In mammals, including humans, ingested arsenic in the form of iAs, is rapidly metabolized and excreted, primarily in the urine, mostly in the form of DMA^V (El-Masri & Kenyon, 2008; Hughes et al., 2008; IARC 2004, 2012; Kenyon et al., 2008a; Le et al., 2000, 2004; NRC, 1999). A small portion is excreted as MMA, and in rodents significant amounts of TMA^VO are formed and excreted in urine (Cohen et al., 2006a). A small portion of ingested iAs is excreted through the bile into the feces (Csanaky & Gregus, 2005; Cui et al., 2004). Ingested arsenate is rapidly reduced enzymatically to arsenite, either in the gastrointestinal (GI) tract, or in the blood and liver (Aposhian, 1997; Nemeti et al., 2012; Pinyayev et al., 2011). Furthermore, dissimilatory reduction of arsenate, which is the reduction of iAs^V to iAs^{III} by bacteria to obtain energy, and the subsequent excretion of the iAs^{III} as waste, occurs in the anaerobic milieu of the GI tract of various mammalian species, including humans (Herbel et al., 2002). Even though orally ingested arsenate is reduced to arsenite, there are minor quantitative differences between the effects of administered arsenate and those of administered arsenite. These differences are probably due to the kinetics that are involved in the conversion of arsenate to arsenite (El-Masri & Kenyon, 2008; Hughes et al., 2008; Kenyon et al., 2008a,b; Thomas, 2007). Ingested methylated

arsenicals, MMA^V and DMA^V, are mostly excreted unchanged in humans but can be metabolized to DMA^V, DMA^{III} and TMA^VO in rodents (Cohen et al., 2006a). There is no evidence for bioaccumulation of iAs in tissues except for DMA^{III} binding to rat hemoglobin (Lu et al., 2007b) and macromolecular binding resulting in intracellular inclusions in the mouse and human urothelium (Dodmane et al., 2013b; Suzuki et al., 2008a; Wedel et al., 2013) and possibly other tissues (Basu et al., 2004).

The enzyme arsenic (+3 oxidation state) methyltransferase (As3mt) is the major methylating enzyme in mammalian species (Drobna et al., 2009; Hughes et al., 2010; Thomas, 2007). In rodents, this enzyme is capable of adding 1, 2 or 3 methyl groups to trivalent arsenic, forming MMA^V, DMA^V and TMA^VO. In rodents, especially in rats, a significant portion of the ingested arsenic is converted to TMA^VO, and is excreted in the urine (Aposhian, 1997; Thomas, 2007). In all species, a small amount of the arsenic is excreted in the inorganic form, predominantly as arsenite. However, the majority of the arsenic is excreted in the form of its methylated metabolites MMA and DMA (both in trivalent and/or pentavalent forms). DMA is nearly always the major urinary metabolite, but the ratio of MMA to DMA (trivalent and pentavalent together) varies between individuals. Chen et al. (2003a,b) suggested that a higher ratio of MMA to DMA that is excreted in the urine correlates with an increased risk for the development of bladder cancer. In addition, Del Razo et al. (1997) found an increased urinary ratio of MMA to DMA in individuals with cutaneous changes indicative of arseniasis. The mechanistic implications of these findings are unclear, although they suggest that MMA^{III} plays a critical role compared to DMA^{III}. As we suggest later, it is the total trivalent arsenical load that is significant. Genetic polymorphisms in the human gene *as3mt* may affect the quantitative ratio between the various methylated species. It is unclear yet whether this has a significant impact on cancer risk (Engström et al., 2011; Sampayo-Reyes et al., 2010; States et al., 2011; Sumi et al., 2011; Valenzuela et al., 2009). Other methyltransferases, such as N-6 adenine-specific DNA methyltransferase 1 (N6AMT1) (Ren et al., 2011) may also contribute to the methylation of arsenicals, although their quantitative contribution *in vivo* has not been determined.

The extent by which these polymorphisms affect the variability in humans' biological response to arsenicals is an area of ongoing investigation. A recent study (Yager et al., 2013) evaluated genomic expression in primary uroepithelial cells from 15 human donors; the cells were exposed to trivalent and pentavalent arsenicals *in vitro* at ratios comparable to what is found in urine. The authors determined that the range in the benchmark doses for eight genes with a statistically significant dose-response trend varied by only 3-fold. Thus this study found relatively limited variablility in responsiveness. Furthermore, comparison of wild type mice to As3mt knockout mice (where the knockout mice produce virtually no methylated arsenical), indicates similar toxicological responses between wild type and knockout mice, including a similar NOEL (Yokohira et al., 2010, 2011). This is likely due to the contribution of iAs^{III} (versus methylated arsenicals) under conditions when methylation is inhibited. Thus, these studies suggest that the impact of polymorphisms on overall risk from inorganic arsenic and its methylated metabolites is likely to be small.

In humans, the potential for methylation of DMA^V to TMA^VO exists, but does not occur readily (Aposhian, 1997; Cohen et al., 2006a; Thomas, 2007; Waters et al., 2004), and normally results in undetectable amounts of TMA^VO in human urine. Therefore, most of the DMA^{III} that is formed from reduction of DMA^V is spontaneously oxidized back to DMA^V and excreted in the urine. The potential for formation of TMA^VO in humans is evidenced by the presence of TMA^VO in the urine of individuals that were exposed to extremely high levels of arsenic (Marafante et al., 1987; Xu et al., 2008).

The proportion between pentavalent arsenic and trivalent arsenic in the total MMA and DMA in urine is uncertain due to methodological problems in the analytical procedures for determination of MMA^{III} and DMA^{III} (see the section titled "Definition of the arsenic species analyzed"). The trivalent forms of organic arsenic, MMA^{III} and DMA^{III}, have been detected in the urine of rodents and humans that were exposed to high concentrations of arsenicals (Le et al., 2000, 2004; Valenzuela et al., 2005).

Two additional possible metabolic pathways of iAs have been proposed during the past decade, which may explain some of the differences in metabolism between species: (a) glutathione conjugation, and (b) thiolated analogs.

(a) Glutathione conjugation: Hayakawa et al. (2005) suggested an intriguing hypothesis, that methylation of arsenicals occurs through arsenic-glutathione conjugates, which implies that glutathione is essential for the activation of the enzyme arsenic methyltransferase. Using human recombinant arsenic methyltransferase (Cyt19) *in vitro*, these researchers demonstrated a pathway in which methylation of arsenicals occurs through certain arsenic-glutathione complexes that have been identified in bile *in vivo*. However, according to Thomas et al. (2007, 2009, 2010), a glutathione-dependent pathway plays a minor role, if at all, in potentiating the methylation reaction that is catalyzed by arsenic methyltransferase, and is not required for the reaction to take place. Furthermore, the existence of the intermediates described by Hayakawa et al. (2005) has been questioned from a chemical point of view by Cullen (personal communication). Taken together, the evidence suggests that this alternative pathway for arsenic methylation does not contribute significantly to arsenic metabolism, if it exists at all in the whole organism.

(b) Thiolated analogs: This recently discovered, biochemical metabolic process which appears to be critical for our understanding of arsenic metabolism and kinetics, as well as for its effects, involves production of thiolated analogs of oxygen-containing methylated metabolites of inorganic arsenic (Fricke et al., 2005; Naranmandura et al., 2008; Raml et al., 2007; Thomas, 2010). These thiolated arsenicals have been identified as mono-thiol and di-thiol forms of MMA^V and DMA^V and thiolated analogs of iAs (Figure 2) (Pinyayev et al., 2011). It was hypothesized originally that these thiol metabolites were generated following the interaction of the trivalent forms of arsenic with sulfhydryl groups in proteins or smaller molecules,

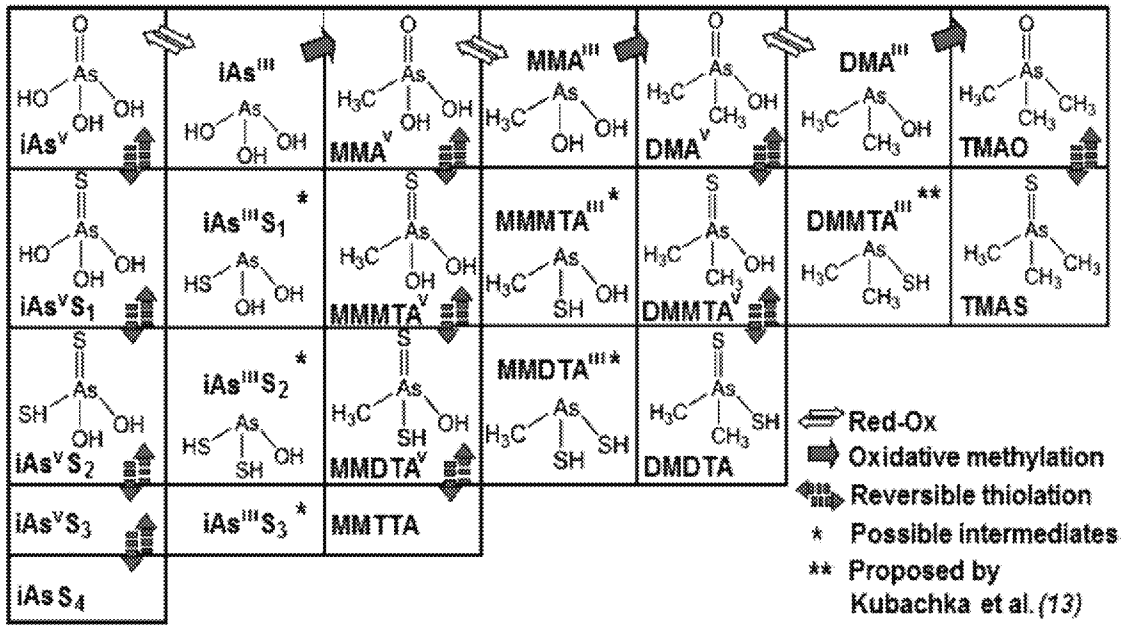

Figure 2. Structure and metabolic conversion of oxyarsenicals to thiolated arsenicals by interaction with hydrogen sulfide (from Pinyayev et al., 2011). Used with permission.

such as glutathione, with ultimate cleavage of the sulfhydryl groups from the peptide, leading to the formation of the thiol metabolites (Naranmandura et al., 2008). However, recently it has been shown that a major source of the thiol metabolites, if not the only source, may be the reaction of iAs^{V}, MMA^{V} and/or DMA^{V} with hydrogen sulfide (H_2S) in the anaerobic milieu of the GI tract, and possibly in the mammalian cells themselves (Figure 2) (Fricke et al., 2005; Pinyayev et al., 2011; Rehman & Naranmandura, 2012; Thomas, 2007, 2009, 2010). The formation of the thiol arsenicals from H_2S does not appear to require enzymatic activity. The thiol arsenicals generally constitute only a small portion of the total amount of arsenic in any given biological setting based on studies showing approximately 5% or less in humans and less than 1% in rats (Raml et al., 2007; Suzuki et al., 2010). They are probably not readily oxidized to the corresponding oxyarsenicals while in the GI tract (Pinyayev et al., 2011), and provide some interesting features, which probably contribute to the overall biologic activity of inorganic arsenic in the body (Pinyayev et al., 2011; Rehman & Naranmandura, 2012). Some of the thiolated arsenicals have been shown to be cytotoxic *in vitro* to urothelial cells (Naranmandura et al., 2011; Suzuki et al., 2010), to lung epithelial cells (Bartel et al., 2011; Chilakapati et al., 2010) and to epidermal keratinocytes (Naranmandura et al., 2007). The thiolated pentavalent arsenicals cross cell membranes more rapidly than their oxygenated analogs (Pinyayev et al., 2011; Rehman & Naranmandura, 2012; Suzuki et al., 2010). Once inside the cell, they are rapidly converted to the corresponding trivalent, oxygen-containing form of arsenic (Suzuki et al., 2010). This rapid transport into the cell and the conversion to trivalent forms of arsenic provide the explanation for the significant cytotoxicity of these thiol metabolites compared to the pentavalent oxygen-containing arsenicals.

Toxicokinetics of inorganic arsenic in mammalian species

Epidemiological and experimental studies show that humans are more susceptible to the carcinogenic effect of iAs than rodents, and more susceptible to acute toxicity of iAs. The differences in susceptibility are probably due to differences in metabolism and toxicokinetics between the species (US EPA, 2007). Following identical oral exposures, the systemic concentration of trivalent arsenicals in humans (in terms of ''mg/kg bw'' basis) has been suggested to be higher than the systemic concentration in rodents, although evidence for direct comparisons is not available (Adeyemi et al., 2010; El-Masri & Kenyon, 2008; Kenyon et al., 2008a,b). The differences in toxicokinetics include, among others, variations in transport, cellular interactions and sequestration.

Transport

A number of cell membrane transport systems have been identified for arsenicals. For arsenate, the major transport systems are various phosphate transporters, such as the sodium/phosphate co-transporters (Villa-Bellosta & Sorribas, 2010). The major transporters for the trivalent arsenicals include P-glycoproteins, ATP-binding cassette transporters (multidrug-resistant proteins), and glucose transporters (GLUT and SGLT) (Calatayud et al., 2010, 2012; Carbrey et al., 2009; Chavan et al., 2011; Drobna et al., 2010; Jiang et al., 2010). Trivalent forms of the oxygen-containing methylated arsenicals are readily transported across the cell membrane (Drobna et al., 2010; Thomas, 2007), whereas the methylated pentavalent compounds are not easily transported. In contrast, the thiolated compounds of arsenic, both trivalent and pentavalent, cross the cell membrane readily (Suzuki et al., 2010). The specific transporters involved with the transport of the thiolated arsenicals have not been identified. Of critical importance for evaluating comparisons of effects between specific tissues and specific animal species

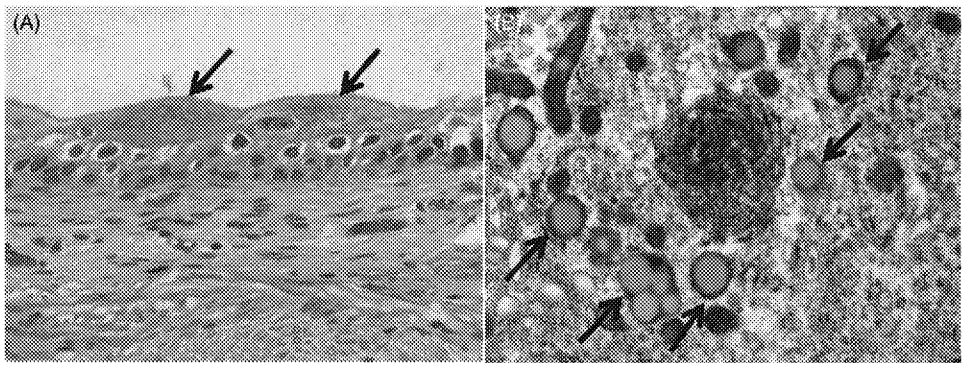

Figure 3. (A) Light microscopic section of urinary bladder urothelium from a mouse treated with 100 ppm arsenite in the drinking water for 4 weeks showing numerous intracytoplasmic inclusions (arrows) in superficial cells. (B) Electron micrograph showing round, amorphous inclusions (arrows) in mitochondria of superficial cells from a mouse treated with arsenite in the drinking water for 4 weeks. These were purified to demonstrate their presence in mitochondria and analyzed to show that they contained arsenic in the form of iAs^{III} bound to protein.

are: (a) the marked variation in the affinity of the transporters for the different arsenicals, (b) the specificity of the variants of each of these transporters to the various tissues, and (c) the marked variation in the quantitative aspects of the various transporters among animal species, including humans (Calatayud et al., 2012; Drobná et al., 2010; Thomas, 2007).

Cellular interactions

The biological activity of arsenicals depends on the formation of the trivalent forms and their interaction with sulfhydryl groups of proteins or small peptides such as glutathione, or of other small molecules such as lipoic acid and cysteine (Cullen, 2008; Kitchin & Wallace, 2008; Schmidt & Mickein, 2011, 2012). The differences between effects of arsenicals in different species are due to multiple variances in toxicokinetics, among which are differences in the cellular uptake of the various arsenic compounds, the rate of metabolism, and the rate of interaction with the target sulfhydryl groups.

There is a significant variation in the structure and content of amino acids in specific proteins of different species, affecting the availability of sulfhydryl groups, which may produce variability in the reactivity of arsenicals for various tissues and between species. For example, rat hemoglobin binds DMA^{III} to a specific cysteine in the alpha chain that is not present in other species (Lu et al., 2007b). Binding to the estrogen receptor of mice may similarly be species specific (Kitchin & Wallace, 2008).

Sequestration

A substantial portion of iAs that is administered to rats is rapidly sequestered in red blood cells (Aposhian, 1997). Lu et al., (2007b) demonstrated that the sulfhydryl group of cysteine 13 in the alpha chain of rat hemoglobin binds arsenic in the form of DMA^{III}. This specific cysteine is not present in the hemoglobin alpha chain of other species. *In vitro*, arsenite and MMA^{III} also bind to this rat hemoglobin sulfhydryl group, but DMA^{III} is the only form that binds to the rat hemoglobin *in vivo*, whether inorganic arsenate, MMA^{V}, or DMA^{V} were administered. As explained earlier, this is just one example of the limitations of *in vitro* studies and caution is necessary when extrapolating *in vitro* findings to *in vivo* situations.

Suzuki et al. (2008a) observed that in mice, arsenicals are sequestered in the form of intracytoplasmic, intramitochondrial inclusions in the urothelial cells (Figure 3). The inclusions, which accumulate over time in direct relationship to dose, contain arsenic, almost entirely as trivalent iAs, which is bound to macromolecules. By electron microscopy, these inclusions are stained as though they contain lipids, suggesting the possibility that the proteins involved could be lipoproteins, although this has not yet been chemically characterized.

We recently identified by light and electron microscopy, similar intracytoplasmic inclusions in exfoliated urothelial cells from patients who had been treated with high doses of arsenic trioxide for promyelocytic leukemia (PML) (Figure 4). Examination of these cells by light microscopy (Wedel et al., 2013) strikingly revealed that these inclusions have the same morphological appearance as reported to be found in urothelial and buccal squamous cells and in lymphocytes from individuals in populations that were exposed to high concentrations of iAs in drinking water (Basu et al., 2004). These inclusions were previously interpreted to be micronuclei (Basu et al., 2004). Non-specific staining procedures that were used for detection of micronuclei have been the source of the mischaracterization in a number of circumstances, not just with iAs (Nersesyan et al., 2006). When centromeric staining, which is more specific for DNA, is used, the increased inclusions are clearly recognized as centromere negative (Smith et al., 1993; Ghosh et al., 2008). When specific staining procedures for light microscopy, as well as electron microscopy are used, these inclusions were demonstrated not to be micronuclei. These inclusions are not present in rats (Suzuki et al., 2008a), which corresponds to the observation that ''micronuclei'' have not been detected in rats administered high doses of arsenicals (Wang et al., 2009). Thus, it is very likely that the ''micronuclei'' that were reported to be observed in humans that had been exposed to iAs (Moore et al., 1997) were actually the same intracytoplasmic inclusions we identified in mice and found not to be micronuclei. Therefore, all previous reports of micronuclei in subjects exposed to iAs are questionable until a revised examination is conducted to determine whether they are truly micronuclei, or if they are actually intracytoplasmic inclusions of arsenic. As mentioned

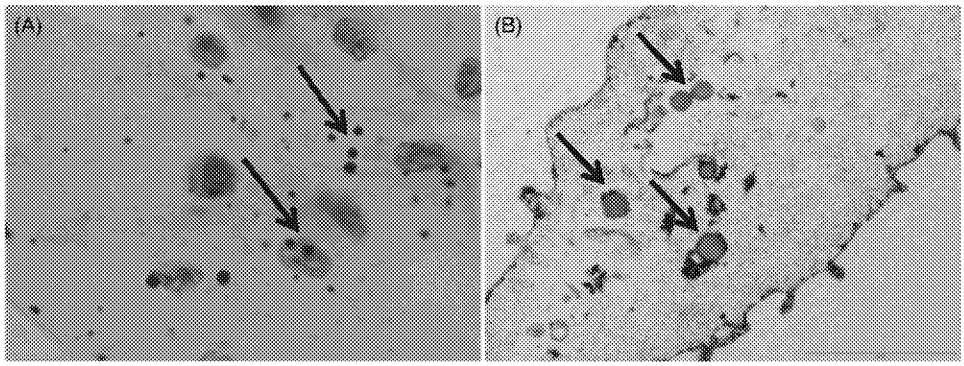

Figure 4. (A) Light microscopic appearance of exfoliated superficial cells in the urine from a patient with promyelocytic leukemia treated with arsenic trioxide. Several intracytoplasmic inclusions (arrows) are present. (B) Electron micrograph showing round, amorphous inclusions (arrows) in membrane-bound organelles, possibly lysosomes.

above, similar inclusions have been reported in various tissues in humans and other species exposed to various metals, such as lead (Brown et al., 1985; Gonick, 2011; Masci & Bongarzone, 1995; Nersesyan et al., 2006). These arsenic-containing inclusions do not have an adverse effect on the cells, but appear to serve as a defense at high exposures, similar to lead and other metal inclusions. These inclusions were not observed in humans not exposed to high levels of iAs (Wedel et al., 2013).

Sequestration has not been observed in hamsters. In fact, the overall metabolism and kinetics of arsenic in the hamster are more similar to those of humans than to those of rats or mice, although $TMA^{V}O$ forms readily in hamsters, unlike in humans (Aposhian, 1997). It is clear that sequestration is one of the factors that contribute to the differences in toxicokinetics between species.

Carcinogenicity of arsenicals in rodents

Inorganic arsenic was not considered to be carcinogenic to rodents until 2001, because carcinogenicity studies of iAs in rodents, by any treatment route, whether administered as arsenite or arsenate, did not show increased incidences of tumors (Tokar et al., 2010b). During the past decade, several models have been developed demonstrating carcinogenicity of various forms of arsenic in rodents (Cohen et al., 2006a, 2007; Tokar et al., 2010a,b; 2011; Waalkes et al., 2003). Since new animal models of arsenic carcinogenicity have been developed, it is no longer appropriate to conclude that rodents are resistant to the carcinogenicity of arsenicals and that studies with rodents when appropriately designed and interpreted are not relevant to humans. The following sections describe the carcinogenicity studies of various arsenic compounds in rodent models.

Carcinogenicity studies with MMA and DMA in rodents

Monomethylarsonate (MMA^{V}) was administered to rats and mice in two-year bioassays, resulting in no carcinogenicity (Arnold et al., 2003). The absence of an increased incidence of tumors was further supported by the lack of pre-neoplastic proliferative lesions (Arnold et al., 2003). These studies provide evidence for no carcinogenic risk of MMA^{V} to humans.

Recently, Tokar et al. (2012) reported an increased incidence of liver, adrenal and lung neoplasms in male mice, and of uterine, adrenal, and ovarian neoplasms in female mice in which the dams were treated with MMA^{III}. The chemical was administered to dams during gestation days 8 to 18, following the model established by Waalkes (Waalkes et al., 2003) for transplacental studies with iAs.

High doses of DMA^{V} ingested by rats in the diet or in drinking water produced low incidences of tumors in the rat urinary bladder in two-year bioassays (Arnold et al., 2006; Wei et al., 1999, 2002). The effect appeared to be greater in female rats than in males. The no observed effect levels (NOEL) following exposures in the diet and in drinking water were similar. When the administration was through the diet, tumors were induced at a concentration of 100 ppm, with a hyperplastic response at 40 ppm. No tumors or hyperplasia were seen in females treated with 2 or 10 ppm of DMA^{V} in the diet for two years (Arnold et al., 2006; Cohen et al., 2006a, 2007). More sensitive techniques showed urothelial effects in rats treated with a dose of 10 ppm, but no effects were seen in rats treated with 2 ppm in the diet (Cohen et al., 2006a). Likewise, an increased incidence of bladder tumors was seen in male rats that were administered 50 or 200 ppm DMA^{V} in the drinking water, but no tumors or hyperplasia were observed in rats treated with 12.5 ppm DMA^{V} in drinking water (Wei et al., 1999, 2002).

No tumors or hyperplastic effects were observed in mice treated for 2 years with up to 500 ppm DMA^{V} (Arnold et al., 2006), in hamsters that were administered up to 100 ppm DMA^{V} in the diet for 10 weeks (Cano et al., 2001), as well as in other animal models, including OGG-1 knockout mice (deficient in oxidative damage repair) (Cohen et al., 2006a), ornithine decarboxylase (ODC) knockout mice (investigated for skin carcinogenesis), and various other strains of mice investigated for possible lung carcinogenicity (Cohen et al., 2006a). All of these studies showed essentially no statistically significant increase in tumor incidence or in hyperplasia following administration of DMA^{V} at any dose. Furthermore, dietary administration of DMA^{V} did not induce preneoplastic or cytotoxic changes in short term studies in hamsters, performed at similar doses and times that gave positive results in rats (Cano et al., 2001) The conclusion is that DMA^{V} is a rat specific bladder carcinogen.

Administration of methylated arsenicals with other agents in two-stage studies has generally corroborated the carcinogenic findings of the same agents when administered alone (Tokar et al., 2010a,b). For example, pretreatment with N-butyl-N-(4-hydroxybutyl) nitrosamine (BBN), followed by administration of DMA^V in the drinking water led to a marked increase in bladder tumors (Wanibuchi et al., 2004). Also, an increase in incidence of tumors in the urinary bladder, kidney and liver were demonstrated in a study involving a complex pretreatment with five different DNA-reactive carcinogens (Wanibuchi et al., 2004). However, as discussed by Cohen et al. (2006a), this protocol was complicated, with marked alterations in metabolism and toxicokinetics of the subsequently administered agents, so that interpretation of the results is difficult, if not impossible.

Carcinogenicity of inorganic arsenic in rodents

Waalkes et al. (2003) had a signifcant breakthrough in the study of iAs carcinogenicity using a transplacental protocol in mice. Since gestation is often a period of high sensitivity to chemical carcinogenesis, these researchers administered 42.5 and 85 ppm arsenite in drinking water *ad libitum* to pregnant mice, during days 8 to 18 of gestation. Dose-related increases in tumor incidence were observed in the liver, lung, adrenal, and ovary tissues of the offspring, after they had reached adulthood. The development of this animal model was a major advance in the study of iAs carcinogenesis. However, the results from this model must be interpreted with caution. For example, two of the tumor types, adrenal and ovary, have not been found to be associated with iAs in humans. Furthermore, in follow-up experiments with the transplacental mouse model, an inhibitory effect of arsenite was found on formation of liver tumors (Ahlborn et al., 2009; Nelson et al., 2009). The adrenal and ovary tumors, and possibly the liver tumors, might be related to non-cancer effects on hormones, such as estrogen, rather than to a direct effect of arsenic on the specific tissues. Kitchin & Wallace (2005) have shown that trivalent arsenicals can interact with a free sulfhydryl group in the mouse estrogen receptor. The lung tumors are discussed further in the section on lung cancer.

In a life-long study with rats from the Ramazzini Institute, a single bladder carcinoma was detected (Soffritti et al., 2006). Although studies from this Institute related to pulmonary infections and findings related to lymphomas have been criticized (see for example, NTP, 2011), the finding of lesions in the bladder is unlikely to be subject to some of the same criticism and may represent a treatment-related finding. A similar low number of bladder tumors in a two year study in mice administered arsenite was recently reported by Waalkes' group (Tokar et al., 2011).

In contrast to these effects following transplacental exposure, treatment of mouse pups beginning after birth or weaning did not induce similar effects (Tokar et al., 2010a,b, 2011). However, when mice that had been exposed during gestation were subsequently administered diethylstilbestrol (DES) or tamoxifen (not bladder carcinogens by themselves), there was an increase in the incidence of high grade, malignant urogenital epithelial tumors as well as in the number of liver tumors (Waalkes et al., 2006).

In a recently reported experiment, arsenite was administered to CD1 mice at doses of 6, 12 or 24 ppm in the drinking water, prior to breeding, during gestation and lactation, and then to the offspring, beginning and continuing after weaning, for 2 years. The observed distribution of tumors was similar to that observed when the arsenite was administered only transplacentally, although the tumor incidences were higher in the lung, liver, adrenals, uterus, and ovaries, than in mice treated only transplacentally (Tokar et al., 2011). There were also proliferative lesions observed in the urinary bladder, including a single papilloma and an increase in hyperplasia. In this study, the urothelial tumor incidence was not statistically significant, but it should be considered potentially treatment-related, given the rarity of these tumors in the CD1 mouse strain and the presence of urothelial hyperplasia in a significant number of the treated mice.

Since iAs is known for its association with skin changes, including skin cancer in humans, Tokar et al. attempted to generate a model for skin carcinogenesis (Tokar et al., 2010a,b). Administration of arsenite by itself, either orally or directly to the skin, resulted in no effect in wild type mice or in models that are extremely sensitive to induction of skin carcinogenesis, such as SKI (hairless) mice or Tg.AC homozygous mice. Mixed results were obtained from two-stage studies in which arsenite or arsenate was administered together with, or following treatment with known carcinogens such as UV-radiation or 9,10-dimethyl-1,2-benzanthracene (DMBA). In some experiments a decrease in latency (time of development of the first tumor) and an increase in the number of tumors per mouse were observed, but no increase in the incidence. There is no definitive evidence that arsenic increases skin cancer incidence in mice, whether administered orally or topically through the skin (Tokar et al., 2010b).

The tumorigenic effects of exposure to arsenicals by inhalation have not been routinely studied. Furthermore, rodent lung is not a satisfactory model for human lung carcinogenesis, as the findings do not generally correlate with the types of tumors and other lesions, seen in humans following exposure to arsenic. The National Toxicology Program (NTP, 2000) performed an inhalation study with gallium arsenide in rats and mice. Gallium arsenide was studied because of its widespread use in the microelectronics industry, the potential for worker exposure, and the absence of chronic toxicity data. Male and female F344/N rats and B6C3F1 mice were exposed to gallium arsenide particles by inhalation for 16 days, 14 weeks, or 2 years. The exposure to the particles caused hyperplasia of the tracheobronchial lymph nodes secondary to the respiratory tract inflammation and a spectrum of non-neoplastic inflammatory lesions in the lung of rats and mice and in the larynx of male rats. There was no evidence of carcinogenicity in mice and limited evidence in rats. Female rats had an increased incidence of lung adenomas but not carcinomas, and male rats did not show an increased incidence of any tumors. The lung adenomas appear to be associated with the particles and inflammation rather than with the specific chemical (Bomhard et al., 2013). An increase in adrenal medullary pheochromocytomas was also observed in this study, but the relevance of such tumors in the Fisher rat to humans is questionable, especially since they have frequently been observed in rats with particle-induced

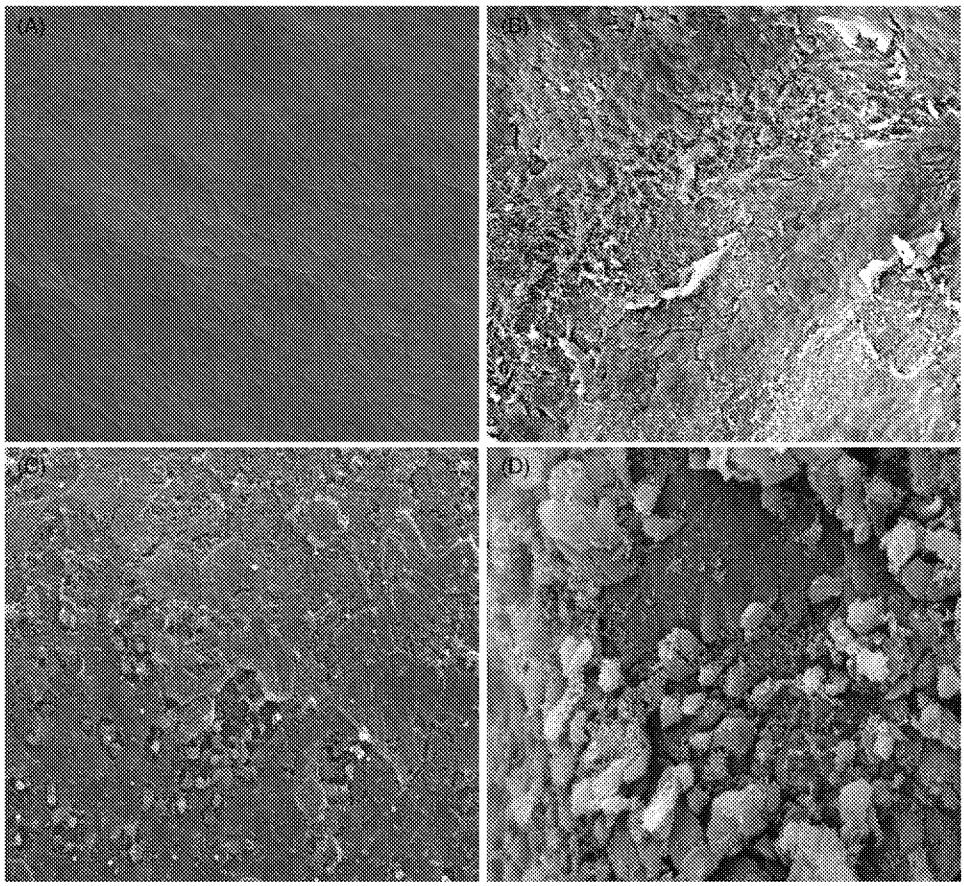

Figure 5. (A) Scanning electron micrograph (SEM) of normal rat urothelium, showing a flat surface of large polygonal cells tightly adherent to each other. (B) SEM of urothelium from a rat treated with 100 ppm DMA^V showing extensive superficial necrosis and exfoliation of superficial cells focally revealing underlying intermediate cells. (C) SEM of urothelium from rat treated with 100 ppm of arsenite in the diet for 4 weeks showing similar changes to those rats treated with DMA^V. The same changes are seen with arsenite or arsenate administered in the diet or drinking water to male or female rats. (D) SEM of urothelium from mouse treated with 50 ppm of arsenite in the diet for 1 week showing similar changes as in the rat. The same changes are seen with arsenate or arsenite administered in the diet or drinking water to male or female rats.

pulmonary inflammation, regardless of chemical composition (Greim et al., 2009). The studies with gallium arsenide do not contribute to our understanding of iAs carcinogenesis (Bomhard et al., 2013).

Calcium arsenate, administered by intratracheal applications to hamsters, induced a slight increase in lung adenomas, with no increase in the incidence of malignant tumors in one study, but with an increase in a subsequent one (Tokar et al., 2010b). Interpretation of the results of these studies was complicated by high mortality rates due to the intratracheal administration.

Mode of action of arsenicals in rodents

Mode of action of DMA^V

As described earlier, DMA^V administered in the diet or in the drinking water to rats in two year bioassays, produced urinary bladder cancer (Cohen et al., 2006a). When the administration was via the diet, the No Observed Effect Level (NOEL) for any urothelial change was 2 ppm. Rats treated with 10 ppm of DMA^V showed cytotoxic changes and increased proliferation, albeit at incidences that are not statistically significant (Figure 5). Similarly, rats treated with 12.5 ppm DMA^V in the drinking water showed no increase in tumor incidence and no cytotoxic or proliferative changes (NOEL = 12.5 ppm).

Extensive follow-up studies revealed that the MOA for the formation of DMA^V-induced tumors of the urinary bladder in rats involves the following key events (Cohen et al., 2006a):

(1) Ingestion of relatively large amounts of DMA^V,
(2) Reduction of DMA^V to DMA^{III},
(3) Renal concentration of DMA^{III} and its excretion in the urine,
(4) Urothelial cytotoxicity,
(5) Regenerative urothelial proliferation, and
(6) Development of urothelial tumors.

Trivalent arsenicals were shown to be highly cytotoxic in several *in vitro* studies with rat urothelial cells, even at concentrations lower than 1 μM, whereas the cytotoxicity of the pentavalent methylated arsenicals was in the millimolar range (Cohen et al., 2006a). Thus, the key to the MOA of DMA^V bladder carcinogenicity in rat is the formation and excretion in the urine of substantial amounts of the highly cytotoxic metabolite, DMA^{III}. Demethylation does not occur in mammals (Aposhian, 1997; Thomas, 2007, 2009, 2010). When DMA^V is administered to experimental mammals, the forms of arsenic that are excreted in the urine are DMA^V, DMA^{III}, and TMA^VO, along with relatively low concentrations of two thiolated arsenicals (reviewed in Cohen et al., 2006a). Although DMA^{III} is a minor metabolite, concentrations greater than 1 μM have been detected in the urine of rats

administered 100 ppm of DMA^V in the diet (Cohen et al., 2002, 2006a). Measurable amounts of DMA^{III} were detected in rat urine when DMA^V was administered in the diet at doses of 10 ppm or higher. However, when DMA^V was administered to rats at a dietary dose of 2 ppm, DMA^{III} was undetectable in the urine, using an analytical method for DMA^{III} with a detection limit of 0.026 μM for DMA^{III} (Cohen et al., 2002; Cohen et al., 2006a; Lu et al., 2003). According to this rat model, there is a close correlation between the formation of DMA^{III}, its concentration in urine, and the induction of urothelial cytotoxicity (Figure 5), with consequent regenerative proliferation. The *in vivo* findings correlated closely with *in vitro* cytotoxicity assessments. Based on these sets of experiments with DMA^V in rats that characterize the key events in tumor formation, the USEPA and the EPA Science Advisory Board concluded that the dose response of DMA^V was non-linear with a threshold, and that the MOA for DMA^V-induced bladder cancer in rats was relevant to humans only when they are exposed to doses of DMA^V that are sufficiently high to lead to cytotoxic levels of DMA^{III} in the urine, and that it is unlikely that such urinary levels could be reached during normal use of the product (US EPA, 2007). This is particularly true based on the more recent finding that DMA^V is a poor substrate for the enzyme As3mt in humans (Thomas, 2007); thus, it would be expected to be activated less in humans than in rats, the species in which tumors were identified.

In vitro, urothelial cells are more sensitive to the cytotoxic effects of chemical agents than *in vivo*, because *in vivo* there is a full differentiation of the urothelium with formation of a superficial protective urothelial cell layer, which acts as a barrier to compounds between the urine and the urothelial cells (Wu et al., 2009). The *in vitro* data for DMA^{III} indicate that there is a minimal, threshold concentration that is necessary to produce cytotoxicity of the urothelium. Specifically, *in vitro* studies with urothelial cells from rats and from humans have shown that concentrations of DMA^{III} greater than 0.2 μM can produce cytotoxicity (Cohen et al., 2006a; Gentry et al., 2010; Sen et al., 2007). This concentration is well above the detection limit of the analytical method, which is 0.026 μM. Therefore, if DMA^{III} is not detected in urine using a reliable method, its concentration is below the cytotoxic concentration.

Following the administration of DMA^V to rats, two pentavalent thiolated metabolites were detected in the rats' urine, in addition to DMA^{III} (Cohen et al., 2006a), as described in the section on metabolism. These metabolites are rapidly transported across the cell membrane and converted to the corresponding trivalent oxygen-containing arsenic compounds such as DMA^{III}, which are highly reactive (Suzuki et al., 2010). The pentavalent thiolated metabolites are considerably more cytotoxic *in vitro* than their pentavalent oxygenated analogs, but less potent than the trivalent MMA^{III} and DMA^{III} (Suzuki et al., 2010), and their overall contribution to the cytotoxicity of arsenicals is limited. Evidence suggests that the cytotoxicity induced by the oral administration of DMA^V is caused by binding of reactive DMA^{III} to specific sulfhydryl groups of urothelial proteins, although the specific proteins have not been identified.

Several factors in the metabolism and toxicokinetics of DMA^V in the organism cause the differences between the responses of humans to DMA^V and the responses of rats. The most obvious is the sequestration of DMA in the red blood cells of rats, which does not occur in humans. The two species also differ quantitatively in transport across cell membranes (Cohen et al., 2006a). Rats form and excrete significant amounts of TMA^VO, whereas TMA^VO is undetectable in urine of humans unless they were exposed to extremely high doses of iAs (Marafante et al., 1987; Xu et al., 2008).

Mode of action of inorganic arsenic

The metabolism of iAs is more complicated than that of DMA^V. iAs has a larger number of possible metabolites, with many variations and combinations involving cell transport, metabolism, kinetics, and interactions with specific sulfhydryl groups of different proteins (El-Masri & Kenyon, 2008; Hughes et al., 2008; Kenyon et al., 2008a; Pinyayev et al., 2011; Rehman & Naranmandura, 2012; Thomas, 2010). Nevertheless, there are important similarities between the MOA of DMA^V and that of iAs. In both cases the MOA for carcinogenicity involves the formation of sufficient levels of trivalent arsenicals that are critical to produce effects, and which their binding to critical sulfhydryl groups leads to cytotoxicity, regenerative proliferation, and ultimately to tumors (Clewell et al., 2011; Cohen et al., 2007; Gentry et al., 2010; Suzuki et al., 2010). Supporting evidence for this MOA is accumulating for all three target tissues (skin, lung and urinary bladder), with the most extensive data available for the bladder urothelium.

Mode of action for the formation of urinary bladder cancer

Key events

The postulated MOA for urothelial carcinoma associated with iAs exposure involves the following key events, which are further described in detail:

(1) Ingestion of relatively large amounts of iAs, pentavalent (iAs^V, arsenate) or trivalent (iAs^{III}, arsenite),
(2) Reduction of arsenate to arsenite,
(3) Sequence of methylations and reductions that ultimately leads to the generation of reactive metabolites, including oxygenated compounds of trivalent and pentavalent arsenic and thiolated pentavalent arsenic compounds,
(4) Reaction with sulfhydryl groups of specific proteins in the target tissue,
(5) Cytotoxicity caused by the reactive metabolites (Figure 5),
(6) Regenerative urothelial proliferation (including hyperplasia), and
(7) Development of urothelial tumors.

These key events are depicted in Figures 6 and 7 and described in detail below:

(1) Ingestion:

The first key event is the ingestion of a relatively large amount of iAs in the form of pentavalent inorganic arsenic (iAs^V, arsenate) or trivalent inorganic arsenic (iAs^{III}, arsenite), in the drinking water and/or in the diet. For humans, the necessary concentration appears to be 100–150 ppb in the drinking water and for rats and mice,

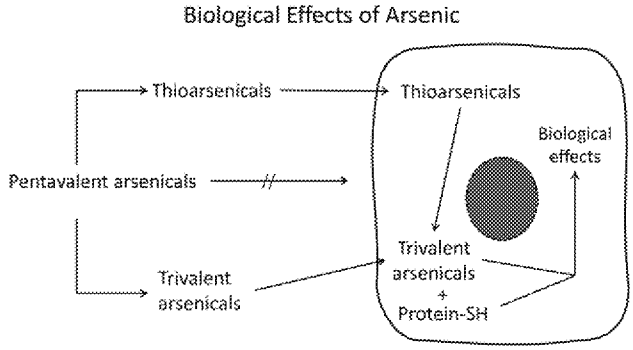

Figure 6. Interaction of arsenicals with cells. Pentavalent arsenicals, except arsenate, are poorly transported across cell membranes. Trivalent arsenicals are efficiently transported across cell membranes. Oxygenated arsenicals can be converted to the corresponding thiolated arsenicals, which can readily enter cells. The thiolated arsenicals inside the cell are rapidly converted to the corresponding trivalent oxyarsenical. Trivalent arsenicals can react with free sulfhydryl groups. If sufficient interactions with specific targets are achieved, a biological response occurs.

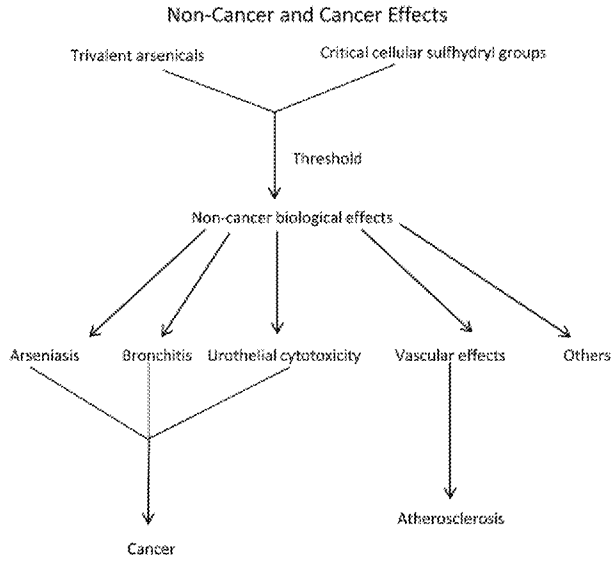

Figure 7. Overall mode of action for biological effects of arsenicals. Various forms of arsenic are converted to trivalent arsenicals which can react with sulfhydryl groups. Depending on the specific sulfhydryl target(s) in specific cells in specific species, if sufficient interactions are achieved, a biological response occurs. Many of these are toxic to the cell. Certain of these non-cancer biologic responses, such as urothelial toxicity, skin arseniasis and chronic bronchitis, involve cell death with consequent regenerative proliferation. As long as the increased proliferation continues, there is an increased probability of progressing to cancer in the respective organs. For other types of cells, particularly non-epithelial cells, other biological toxic responses can occur resulting in other types of toxicity besides cancer. All of these responses have a non-linear dose response, and since a minimum level of interaction with the targeted sulfhydryl groups is required to achieve a biologic, toxicologic response, a threshold is involved.

greater than 1 ppm in the drinking water or diet. Inhalation may be a significant route for human exposure to iAs in certain industries. For other populations, inhalation contributes less than 1% to the overall arsenic exposure, even in locations with low concentrations of arsenic in the drinking water (NRC, 1999).

(2) Reduction of arsenate to arsenite:

Arsenate is rapidly converted to arsenite in the gastrointestinal tract, in the liver and in other tissues, and possibly also in the blood (Aposhian, 1997; El-Masri & Kenyon, 2008; Kenyon et al., 2008a; Thomas, 2007, 2009), as described in the section on arsenic metabolism.

(3) Sequence of methylations and reductions that ultimately lead to the generation of reactive metabolites, including oxygenated trivalent and pentavalent thiolated arsenic compounds.

In most mammals the reduction of arsenate to arsenite is followed by oxidative methylations and reductions generating MMA^{V}, MMA^{III}, DMA^{V}, DMA^{III}, and $TMA^{V}O$. As explained earlier, the amount of $TMA^{V}O$ is substantially higher in the rat than in other species, but is measurable also in other rodents (Cohen et al., 2006a). Humans, under normal conditions, do not generate $TMA^{V}O$ (Aposhian, 1997; Cohen et al., 2006a; El-Masri & Kenyon, 2008; Kenyon et al., 2008a). The oxygen-containing metabolites can be thiolated by chemical interaction with hydrogen sulfide (H_2S), to form thiol-containing metabolites (Pinyayev et al., 2011; Rehman & Naranmandura, 2012).

During the past decade, it has been considered that the toxicity and carcinogenicity of iAs is caused by the reactive methylated trivalent metabolites, MMA^{III} and DMA^{III}, which are more reactive and more toxic than iAs^{III}. However, recent experiments with the As3mt knockout mouse strain clearly demonstrated that iAs^{III} produces cytotoxic effects on the urothelium, even without generation of methylated trivalent arsenicals (Yokohira et al., 2010, 2011). In short term experiments (1–4 weeks), iAs^{III} caused slightly more pronounced effects on the urothelium of the As3mt knockout mice than in the corresponding wild type. This is most likely because the knockout mouse is unable to excrete arsenic as rapidly as the wild type mouse, and it excretes the ingested arsenic mostly as arsenite (Drobna et al., 2009; Yokohira et al., 2011). The conclusion is that in the mouse, and probably also in other species, all trivalent arsenicals, inorganic as well as organic forms, contribute to the cytotoxicity (Clewell et al., 2011; Yager et al., 2013). The contribution of each of the various trivalent arsenicals to the overall toxicity of ingested iAs depends on the relative potency of the specific arsenical, its concentration in the target tissue and its reactivity with specific sulfhydryl groups.

In vitro studies evaluating cytotoxicity established that MMA^{III} and DMA^{III} are two to four fold more cytotoxic than iAs^{III} (Cohen et al., 2007; Suzuki et al., 2010), and the thiolated arsenicals are slightly less potent than the oxidated trivalent arsenicals (Naranmandura et al., 2011). For the urothelium, cytotoxicity is due to the accumulated concentrations of all of these and their overall relative potencies (Yokohira et al., 2010). The predominant trivalent species in the urine of humans, rats, and wild type mice, are the methylated forms of arsenic, especially DMA^{III}. The cumulative toxicity of all of the trivalent forms, together with that of the thiolated pentavalent metabolites, ultimately causes the cytotoxicity and carcinogenicity of iAs (Clewell et al., 2011; Gentry et al., 2010; Nascimento et al., 2008). The involvement of several toxic forms complicates the assessment and analysis of the MOA of iAs, but the overall process is the same as that of DMA^{V}, i.e. generation of reactive trivalent arsenicals.

(4) Reaction with sulfhydryl groups of specific proteins in the target tissue:

Trivalent oxygen-containing species of arsenic, arsenate, and the thiolated pentavalent metabolites are transported effectively and rapidly across the cell membrane, whereas the pentavalent, oxygen-containing methylated forms are transported poorly (Drobna et al., 2010; Pinyayev et al., 2011; Rehman & Naranmandura, 2012; Suzuki et al., 2010; Thomas, 2007). Interspecies differences are due to differences in the toxicokinetics and in the availability of sulfhydryl groups in specific proteins. The trivalent forms of arsenic (iAs^{III}, MMA^{III} and DMA^{III}) react with sulfhydryl groups that are available on the cell membrane and/or inside the cell (Aposhian, 1997; Jiang et al., 2003; Kitchin & Wallace, 2005, 2008). Thiolated forms, once inside the cell, are rapidly converted to iAs^{III}, MMA^{III} or DMA^{III}, the corresponding oxygen-containing trivalent species, (Suzuki et al., 2010), which react with the proteins' sulfhydryl groups. These include free sulfhydryl groups that are available on proteins and small peptides, such as glutathione, as well as small molecules such as cysteine and lipoic acid. The specific proteins with which the trivalent arsenicals react differ significantly between species and tissues (Kitchin & Wallace, 2005, 2008; Lu et al., 2007b; Yan et al., 2009; Zhou et al., 2011). For example, rat hemoglobin has a cysteine with a free sulfhydryl group in the alpha chain that is not present in other species (Lu et al., 2007b). Another example is the estrogen receptor in mice, which has a free sulfhydryl group that is not available in the estrogen receptor of rats or humans (Kitchin & Wallace, 2005). Such variations in the availability of free sulfhydryl groups on proteins lead to variability between species in biological responses. Furthermore, the reactivity with specific proteins can vary considerably for the different trivalent arsenicals, including reaction with critical regulatory proteins. For example, Rehman et al. (2012) recently demonstrated that MMA^{III} and DMA^{III} react with a tyrosine phosphatase but iAs^{III} does not.

In humans, the generation of arsenic metabolites and their interaction with sulfhydryl groups in specific proteins has been demonstrated by Jiang et al. (2003) and Ngu & Stehdman (2006). This includes specific regulatory proteins such as zinc finger peptides (Demicheli et al., 2011; Zhou et al., 2011), and proteins involved in proliferation, such as Akt and C-Fos (Habib, 2010). Broad proteomic approaches indicate that trivalent arsenicals bind to numerous proteins (Chu et al, 2010; Zhao et al., 2010). It is still unclear which specific proteins are involved in the toxicity and carcinogenicity of iAs in each specific target organ. Binding of arsenite to a specific sulfhydryl moiety in the oncoprotein PML-RAR2 in PML patients is most likely the reason for iAs being clinically useful in treating the disease, and for arsenic trioxide being clinically ineffective in treating other malignancies (Chen et al., 2011; Zhang et al., 2010).

Biological effects depend on the reactivity of the specific trivalent arsenicals with specific proteins. The proteins will vary according to tissue and animal species. The concentration of the arsenical depends on exposure, metabolism, cell transport, and kinetics, which can vary among animal species and among the form of arsenic to which the animal is exposed.

Proteins, in general, have a relatively short half life. The rapid turnover of the specific proteins with which arsenic reacts, and with new proteins rapidly forming to replace the "damaged" ones, is the major reason for the existence of a threshold associated with cytotoxicity. The interaction of trivalent arsenicals below a certain level will not lead to cellular dysfunction. The existence of a pharmacological threshold is clearly demonstrated in PML patients by the requirement of very high doses of arsenic trioxide to achieve clinical efficacy (Dilda & Hogg, 2007; Nicolis et al., 2009).

(5) Cytotoxicity caused by the reactive metabolites:

Cytotoxicity to the urothelium has been demonstrated in rats and mice exposed to high levels of arsenite or arsenate (Suzuki et al., 2008b; Yokohira et al., 2010, 2011) and confirmed *in vitro* by cytotoxicity to urothelial cells of humans and rats. The cytotoxicity occurs both in male and in female rats and mice, but seems to be slightly greater in females than in males, similar to the response of DMA^{V} in rats. Cytotoxicity and cell necrosis appears to be restricted primarily to the superficial cells of the urothelium (Figure 5), while the underlying intermediate and basal cells generally do not undergo necrosis. As the superficial cells become necrotic, they are exfoliated into the urine. The exfoliated sites then start the process of regeneration of basal cells, with ultimate differentiation into intermediate and superficial cells to replace the lost ones (Cohen, 1998).

(6) Regenerative urothelial proliferation (including hyperplasia):

Cytotoxicity and cell necrosis ultimately lead to regeneration, a well-researched process that has been studied in various tissues, such as liver and kidney, and in response to cytotoxicity of a number of toxic substances (e.g. chloroform) (Andersen et al., 2000; Meek et al., 2003). The sequence of cytotoxicity and regeneration is the same for iAs as the sequence that was demonstrated for DMA^{V} (Cohen et al., 2006a, 2007). Numerous other chemicals cause bladder tumors in rodents by the same MOA of cytotoxicity and regenerative proliferation (Cohen, 1998).

As explained before, the metabolism of DMA^{V} is rather simple compared to that of iAs. Following the administration of DMA^{V}, the only trivalent metabolite that is generated is DMA^{III} and it appears that only in rats does this occur to a sufficient extent to produce an effect (Cohen et al., 2006a). This MOA provides a relatively straightforward analysis of the sequence of events, with the key events being metabolism of DMA^{V} to DMA^{III} and interaction of DMA^{III} with specific sulfhydryl groups. Some thiolated arsenicals are also formed, but, as discussed above, are rapidly converted to DMA^{III} in the cells (Suzuki et al., 2010). In contrast, when iAs is administered, the resulting metabolites in the urine, in addition to DMA^{III}, are MMA^{III}, iAs^{III} and thiolated arsenicals, all of which can directly or indirectly react with sulfhydryl groups in the urothelial cells (Suzuki et al., 2010). Since the toxic effect appears to be restricted primarily to the superficial cell layer of the bladder, the increase in regenerative proliferation is relatively mild with a two- to ten-fold increase in BrdU (bromodeoxyuridine) labeling index, and the extent of hyperplasia is generally mild to moderate, as expected from this mild degree of cytotoxicity and necrosis (Suzuki et al., 2008b, 2009).

(7) Development of tumors:

The mild proliferative response is probably the cause for the weak tumorigenic bladder response that was observed in long-term bioassays (Tokar et al., 2010a,b). A similar phenomenon, with a low level of cytotoxicity and proliferation, occurred in rats that were administered high doses of various sodium salts, such as saccharin or ascorbate. Similar to the results of studies with iAs, a standard two-year bioassay with sodium saccharin or ascorbate resulted in increased number of bladder tumors only when the administration period began at birth or *in utero* (Cohen, 1999; IARC, 1999).

In summary, the sequence of events for the generation of bladder tumors in rodents following exposure to iAs involves cytotoxicity, followed by increased proliferation, resulting in the formation of tumors, usually after a long period of exposure.

Mode of action analysis

The proposed MOA is examined using the following Hill criteria that were adapted for the analysis of MOA in animals (Sonich-Mullin et al., 2001):

- Dose-response relationship
- Temporal association
- Strength, consistency and specificity
- Biological plausibility and coherence

These are described in detail as follows:

Dose-response relationship

The short term ($\leq$12 weeks) effects of arsenate and arsenite in mice and rats have been studied extensively (Clewell et al., 2011; Simeonova et al., 2000; Suzuki et al., 2009, 2010; Tokar et al., 2010a,b; Yokohira et al., 2010, 2011). There is a striking consistency among the results of all of these studies regardless of the route of exposure; whether through the diet or through the drinking water; whether in rats or in mice; whether in males or females; and regardless of the endpoint being measured, the NOEL is 1–2 ppm of arsenic (1.73–3.46 ppm of arsenite). Only a small number of tumors were observed, but the induced hyperplasia had a clear dose-response relationship that is consistent with this NOEL. Tokar et al. (2010a,b) administered 6, 12, or 24 ppm of arsenite in the drinking water to mice dams and to pups after weaning. Urothelial hyperplasia was observed in the mice at all three doses, but only one bladder tumor was observed in a male treated with 6 ppm of arsenite. The induction of mild hyperplasia at these relatively early time points is consistent with the weak tumorigenicity seen in the two-year bioassay. Similarly, sodium saccharin fed to rats produces a similarly mild urothelial hyperplasia through 26 weeks of administration and does not produce a significant incidence of bladder tumors in a standard two-year bioassay (Cohen, 1998).

Short term studies in mice and rats showed hyperplasia at 10, 25, 50, and 100 ppm of arsenite in the diet or in the drinking water, with a NOEL of 1 ppm of arsenic (Suzuki et al., 2010; Yokohira et al., 2011). Similar results for mice treated with arsenate were reported by Clewell et al. (2011). Interestingly, a similar dose-response association was detected by scanning electron microscopy (SEM), the most sensitive method available for the detection of morphological changes in the urothelium (Suzuki et al., 2010; Yokohira et al., 2011). Hyperplasia was detected only at doses above the NOEL for cytotoxicity. Interestingly, the reactive arsenic metabolites, including iAs^{III}, and its methylated and thiolated metabolites are detectable in urine only at doses above the NOEL for cytotoxicity (Suzuki et al., 2010). Thus, there is a strong concordance between the dose-response relationship for the generation of reactive arsenicals in the urine and the development of urothelial cytotoxicity. Specifically, there is essentially no detectable change in iAs or MMA levels in blood or tissues (liver, lung, kidney, urinary bladder) until doses exceed 1 ppm As in the drinking water (Kenyon et al., 2008a). In addition, Chen et al. (2011, 2013) showed that as the dose of iAs increased, the increase in the levels of arsenicals in the blood and tissues was not proportional to the increase in dose.

Furthermore, genomic alterations, identified by microarray analysis, showed changes in expression of various genes related to increased cell proliferation, including various gene pathways (Clewell et al., 2011). An extensive review of the literature regarding *in vitro* studies of various arsenicals in a variety of cell systems was conducted by Gentry et al. (2010). These researchers found that trivalent arsenicals at concentrations less than 0.1 μM caused adaptive changes, with no indications of an adverse response. A variety of alterations that were related to various toxicities and increased proliferation occurred at the range of concentrations 0.1–10 μM. Concentrations of trivalent arsenicals above 10 μM were cytotoxic and caused cell death. These findings by genomic analysis are consistent with the morphologic alterations that were identified at the same time points and at the same doses in studies using different methods in mice (Arnold et al., 2013; Suzuki et al., 2010; Yokohira et al., 2011). The apparent wide range of concentrations for these findings is, most likely, due to the variety of cell systems that were tested, the variety of culture conditions, a wide range of times of exposure, treatment with various metabolites, and the measurement of diverse endpoints. In view of these numerous varied factors, the above range of concentrations is not wide at all.

In vitro studies of urothelial cells identified proliferations similar to those observed *in vivo* in various tissues following the administration of arsenate in the drinking water (Clewell et al., 2011). Analyses of gene expression for the dose-response gave similar results *in vitro* (Gentry et al., 2010) and *in vivo* (Clewell et al., 2011). Interestingly, the *in vivo* response in the urinary bladder was also found to be bimodal (i.e. having different responses at different levels of exposure). Mice administered 0.5 ppm of arsenate in drinking water showed adaptive changes, such as stress response, cell adhesion and cytoskeleton remodeling. Gene expression changes were not observed in the urinary bladder of mice administered 2 ppm arsenate. In mice treated with higher concentrations of arsenate (10 and 25 ppm), gene expression changes were evident after one week, concurrent with cytotoxicity that was expressed by initial urothelial changes. Changes after 12 weeks of treatment were consistent with increased proliferation of the bladder epithelium (Arnold et al., 2013; Suzuki et al., 2010; Yokohira et al., 2011).

Cytotoxicity was observed *in vitro* only at concentrations of 0.1–0.2 μM or more of the trivalent or thiolated arsenicals.

No cytotoxicity was observed at concentrations of trivalent or thiolated arsenicals that are lower than 0.1 μM (The analytical method used has a detection level of 0.01 μM.) (Gentry et al., 2010; Yager et al., 2013). This is consistent with the findings from *in vivo* studies in rats and mice, which demonstrated that urothelial toxicity is detected only when the toxic arsenicals are present in the urine at levels above 0.2 μM (Suzuki et al., 2010; Yokohira et al., 2011). The consistency of the findings *in vitro* with those *in vivo* is striking: All of the methods used, including genomic analysis, showed that changes due to urothelial toxicity were detected only at doses at which significant concentrations of the cytotoxic trivalent, or thiolated, arsenicals were excreted in the urine.

In summary, data from multiple studies, *in vitro* and *in vivo*, utilizing a variety of analytical measures and various toxic endpoints, all support the hypothesis that certain, critical levels of reactive metabolites have to be present in the target tissue to induce cytotoxicity (Ahlborn et al., 2009; Clewell et al., 2011; Drobna et al., 2005; Gentry et al., 2010; Suzuki et al., 2010; Tokar et al., 2010a,b; Yager et al., 2013; Yokohira et al., 2011). The association between the presence of a minimum concentration of reactive arsenicals with cytotoxicity, strongly supports the overall hypothesis that the dose-response relationships between exposure to arsenic and toxic effects leading to carcinogenesis are non-linear and have a threshold.

Temporal association

The metabolism of arsenicals begins immediately upon administration of the arsenical, and binding to sulfhydryl groups occurs rapidly. Evidence for cytotoxicity is observed within 6 hours (the earliest time point examined) of administration of effective concentrations (Arnold et al., 2013; Suzuki et al., 2009), and increased cell proliferation is observed shortly thereafter. Hyperplasia is evident as early as seven days after the start of treatment, depending on dose, while development of tumors occurs much later (Tokar et al., 2010a,b). Thus, the temporality of the events is consistent with the sequence of the key events that are postulated for this MOA.

Strength, consistency, and specificity

The evidence supporting a nonlinear MOA *in vivo* and *in vitro* is strong and highly consistent. Numerous studies in several laboratories investigated different aspects of this MOA, utilizing various models, various parameters and various methods of measurement (Arnold et al., 2013; Clewell et al., 2011; Gentry et al., 2010; Simeonova et al., 2000; Suzuki et al., 2008b; Yokohira et al., 2011). The evidence includes results from studies of morphology, cell kinetics, enzymology, metabolism, toxicokinetics, and genomics. The study results are consistent and highly specific for the trivalent arsenicals. The resulting NOEL is approximately 1–2 ppm of iAs in diet or in drinking water, in rats or mice, *in vivo*, while the findings for cytotoxicity *in vitro* are consistently negative at concentrations below 0.1–0.2 μM.

Biological plausibility and coherence

Cytotoxicity and regeneration is a common MOA for the carcinogenicity of non-DNA reactive agents that cause tumors in bladder, lung, skin, and most other tissues (Andersen et al., 2000; Cohen, 1998, 2010; Cohen & Arnold, 2011; Holsapple, et al., 2006) as well as for urothelial carcinogenesis in rodents and in humans (Cohen, 1998). Cytotoxicity can be induced by concentration in the urine of factors that cause either physical or chemical damage to the urothelium. Cytotoxicity and regeneration also contribute to bladder carcinogenesis as seen in humans with bacterial cystitis and schistosomiasis (Cohen et al., 2000). In cigarette smoking related to bladder cancer, the MOA of cytotoxicity and regeneration produced by nongenotoxic substances in the smoke acts synergistically with the genotoxicity from the aromatic amines in the smoke (Cohen et al., 2000).

Cytotoxicity is frequently manifested after metabolic activation of the administered compounds, similar to the case of iAs, which is ingested in the form of arsenate and reduced to arsenite and metabolized to various metabolites. Cytotoxicity as a result of reaction with cellular proteins leads to acute toxicity as well as to carcinogenicity, and can be caused by toxicants such as acetaminophen, which causes liver toxicity, or chloroform, which causes hepatic and renal toxicity and carcinogenesis (Andersen et al., 2000).

In rodents, cytotoxicity of the urothelium can be induced by urinary solids, produced and excreted in the urine and acting as abrasives to the urothelium, or by urinary reactive metabolites, which react with the urothelium, leading to cell death (Cohen, 1998; Cohen et al., 2007). There is no evidence that exposure to inorganic and organic arsenicals leads to the formation of urinary solids (Arnold et al., 1999; Cohen et al., 2007). There is substantial evidence, however, that reactive trivalent arsenicals that are present in the urine are the cause for the cytotoxicity, which appears in response to arsenic administration. Thus, there is strong biological plausibility and coherence of the data supporting the MOA that is postulated for iAs urothelial carcinogenesis.

In summary, analysis by Hill criteria supports the hypothesized MOA. In the following sections the possibility of alternative modes of action is examined.

Examination of proposed alternative modes of action

Several other modes of action have been suggested for iAs carcinogenesis: genotoxicity, direct damage to DNA, indirect damage to DNA, alterations of DNA repair, damage to the mitotic spindle, inhibition of apoptosis, direct mitogenicity, oxidative damage, DNA methylation and immunosuppression. The plausibility of each of the proposed modes of action is examined below. Some of them can be incorporated into the hypothesized mode of action of cytotoxicity and proliferation as associative events, but there is no evidence for others.

Genotoxicity

Genotoxicity is the most extensively investigated alternative MOA for iAs carcinogenesis. Cancer is a clonal disease, therefore, to cause cancer it is critical that several DNA alterations occur in the same pluripotential cell (stem cell) of the target tissue, as described in a review by Cohen & Arnold (2011). It is also clear that tumors develop only after multiple errors have occurred, and that one error in a cell would not lead to a tumor. Errors occur spontaneously every time DNA

replicates. Most of these spontaneous errors are repaired, but not all. Thus, an increased risk of a tumor developing can be either by the agent damaging DNA directly (DNA-reactive) (Cohen et al., 2006b) or when the number of DNA replications increases, leading to an increase in the probability of permanent errors (Cohen & Ellwein, 1990, 1991; Greenfield et al., 1984; Moolgavkar & Knudson, 1981).

When exposed to high levels of arsenicals, DNA replication increases, causing the number of spontaneous errors to increase even if the rate of mistakes per cell division remains the same (Cohen & Ellwein, 1991; Moolgavkar & Knudson, 1981). According to recent evidence, arsenicals cause an increase in the proportion of stem cells in the target tissue (Sun et al., 2012; Tokar et al., 2011; Xu et al., 2012), reflecting the fact that the carcinogenic process involves a gradual transition of the tissue from a differentiating to a proliferating tissue (Cohen & Ellwein, 1991). Errors in DNA replication occur infrequently with each cell division due to the vast and efficient array of DNA repair enzymes that address constant chemical modifications of DNA that are due predominantly to endogenous cellular processes, such as oxidative damage, formation of exocyclic adducts, deamination, interaction with nitric oxide, depurination, and other normal metabolic processes. Such errors take place in each cell hundreds to thousands of times every day, but nearly all of them are repaired. The more replications, the more likely permanent genetic errors will occur. These errors occur even without inhibition of DNA repair, albeit at a low rate.

Based on these precepts, the risk of cancer may increase in one of three situations described as genotoxicity: (i) direct damage to DNA, for example, by DNA-reactive carcinogens; (ii) indirect damage to DNA; or (iii) inhibition of DNA repair mechanisms. Each of these has different implications for risk assessment, as discussed in the following sections:

Direct damage to DNA. Several claims have been made that arsenicals can interact directly with DNA (e.g. Mass et al., 2001). However, interaction with DNA requires reactive electrophiles and in some instances reactive free radicals, whereas iAs and its metabolites have an anionic structure and therefore cannot react with the ionic nucleotides of the DNA. Formation of free radicals, such as peroxy, have also been suggested, but the overwhelming evidence supports a MOA for arsenicals that is non-DNA reactive as arsenic bound directly to DNA has not been demonstrated (Kligerman et al., 2003; Nesnow et al., 2002). Nesnow et al. (2002) demonstrated conclusively that arsenicals do not react directly with DNA, and therefore are not DNA reactive carcinogens.

Indirect damage to DNA. Numerous publications demonstrate that arsenicals cause indirect damage to the DNA (e.g. Kligerman et al., 2003, 2010). Such indirect interactions include micronucleus formation, chromosomal aberrations in *in vitro* assays, and other manifestations of damage to the DNA. Most of these assays are used routinely in the screening for pesticides, pharmaceuticals, cosmetic agents, and other chemicals, but they have little predictive value (<50%) for carcinogenicity, neither positive nor negative (Zeiger, 2001, 2004). Positive results for arsenic in these assays are manifested at cytotoxic concentrations, and therefore cannot serve as an indication for the MOA for arsenic carcinogenesis (see for example, Klein et al., 2007). Cytotoxicity is not always demonstrated in the specific assays, or it is considered low (<10%), but the assays are usually being performed for relatively short periods of time (4-24 hours), which are too short for cell death to be completed. Cell death following toxicity is evidenced by the presence of necrosis, when treatment or observation is extended to longer periods of time (Cohen et al., 2002; Komissarova et al., 2005). Clearly, when cytotoxicity leads to cell death there is damage to the DNA, but since the cell is destined to die, the damage to the DNA is not manifested as carcinogenesis (Cohen et al., 2002). Several aspects of *in vitro* genotoxicity studies raise considerable doubt as to their applicability to *in vivo* situations at exposure levels that can be attained biologically. For example, such high concentrations of trivalent arsenicals usually used *in vitro* could not be reached *in vivo* at environmentally relevant concentrations of iAs. Other aspects include the types of cells used, the duration of the observations (Komissarova et al., 2005) and the types of assays. (See the discussion in the section titled "Precautions in Evaluating Published Research".)

A few studies have reported increased numbers of micronuclei in urothelial cells in urine, in squamous epithelial cells from buccal scrapings, or in lymphocytes from individuals exposed to high levels of iAs in their drinking water or in their occupational environment (Basu et al., 2004; Bartolotta et al., 2011; Martinez et al., 2005; Moore et al., 1997, 2002; Warner et al., 1994). However, the number of micronuclei did not increase in urothelial cells from rats treated with high doses of arsenic (Wang et al., 2009). As discussed earlier, it is very likely that the reported micronuclei in human studies are actually intracytoplasmic arsenic-containing inclusions, like those observed in human urothelial cells from patients treated with arsenic trioxide (Wedel et al., 2013) and in urothelial cells of mice that were exposed to high levels of arsenicals (Suzuki et al., 2008a). Such inclusions were not observed in rats treated with arsenicals (Suzuki et al., 2008a).

Inhibition of DNA repair. An indirect effect of arsenicals on DNA repair, which is expressed as genotoxicity, has been demonstrated in numerous assay systems, both *in vitro* and *in vivo* (Gentry et al., 2010; Kitchin & Wallace, 2008; Kligerman et al., 2010). These *in vitro* studies have frequently been at concentrations of >5 μM of a trivalent arsenical, which are cytotoxic concentrations (Ebert et al., 2011; Gentry et al., 2010; Lai et al., 2011; Ying et al., 2009), and the *in vivo* studies have not shown consistent effects on DNA repair (Clewell et al., 2011; Wang et al., 2009). The effect on DNA repair may result from interaction of trivalent arsenicals with sulfhydryl groups that are available on some of the DNA repair enzymes (Kitchin & Wallace, 2008) and, thus, do not contradict the MOA of cytotoxicity and proliferation. At high arsenic exposures, an inhibition of the functional capacity of DNA repair could occur and potentiate the effects of the cell proliferation occurring in response to the cytotoxicity.

Damage to the mitotic spindle

Interaction of trivalent arsenicals with tubulin, a major protein in the mitotic spindle involved in the mitotic phase of the cell

cycle, has also been proposed as the MOA for the formation of tumors following exposure to arsenic (Kitchin & Wallace, 2008; Li & Broome, 1999). However, damage to tubulin leads to cell death, and is not a heritable change that causes tumors. There are various mitotic spindle poisons, such as colchicine, which are used as therapeutic agents, but they are not carcinogenic.

Inhibition of apoptosis

Inhibition of apoptosis has also been proposed as a possible MOA for the carcinogenicity of iAs. The evidence for an effect on apoptosis comes primarily from *in vitro* studies, but has not been evaluated *in vivo*. Clewell et al. (2011) have conducted genomic analysis and suggested that inhibition of apoptosis may also occur *in vivo* in mice at moderate exposure levels (<2 mg/L), but there was no actual assessment of apoptosis in the urothelium of these mice and the genes involved are known to have other functions.

Inhibition of apoptosis of the urothelium, particularly in conjunction with increased cell replication, would cause an increase of the number of urothelial cells, resulting in hyperplasia, and would allow cells with genetic damage to proliferate. There is indirect evidence that this might actually occur, since in some of the mouse studies there was no increase in the BrdU labeling index, even when hyperplasia was readily apparent by light microscopy and by SEM (Suzuki et al., 2010; Yokohira et al., 2011). The effect of accumulation of cells increases the number of cells that undergo DNA replication during any given time period, even if the rate of proliferation remains the same as that of the controls. The number of DNA replications increases because of the increased number of urothelial cells. If inhibition of apoptosis did occur, it would have an effect only on the accumulation of cells (hyperplasia) that occurred in response to cytotoxicity and cell death. Apoptosis is a rare event in normal urothelium (approximately 1–3 apoptotic cells per 1000 urothelial cells) (unpublished observations), so that inhibition of apoptosis by itself is unlikely to produce a carcinogenic effect. Furthermore, inhibition of apoptosis in the urothelium has not been demonstrated for any chemical (although not many have been examined specifically for this effect), and there is no other evidence that apoptosis inhibition occurs in the urothelium following exposure to arsenicals. Thus, apoptosis can only be considered a potential associative event, not a key event.

Direct mitogenic effect

There is some indirect evidence for a mitogenic effect on urothelial cells *in vitro*, but no direct examination for this effect has been conducted either *in vitro* or *in vivo*. While there have been some studies that have demonstrated increases in cell proliferation as described above, the proliferation was associated with cytotoxicity and subsequent regenerative cell proliferation (Suzuki et al., 2010; Yokohira et al., 2011). Furthermore, time sequence studies in rats and mice administered iAs show that cytotoxicity precedes increased cell proliferation (Arnold et al., 2013) similar to the sequence observed for DMA^V administered to rats (Cohen et al., 2006a). A direct mitogenic effect cannot be distinguished under such circumstances. It is clear that in response to a high iAs dose there is an increase in growth factors, and possibly also in growth factor receptors in urothelial cells, but this increase occurs in conjunction with increase in cell proliferation (hyperplasia) that is always associated with cytotoxicity (Clewell et al., 2011; Suzuki et al., 2010).

Clewell et al. (2011) have applied genomics to demonstrate that changes in gene expression in the urothelium in response to administration of iAs are dose- and time-dependent. Genes that are expressed after one week of administration are primarily associated with cytotoxicity and cell death, whereas changes after 12 weeks are predominantly associated with increased cell proliferation. This finding is consistent with the MOA of cytotoxicity with consequent regeneration, rather than a direct mitogenic effect (Arnold et al., 2013). It is clear that following cytotoxicity, the urothelial cells adapt by increasing cell proliferation, which continues as long as the exposure to iAs continues. Regenerated hyperplastic cells appear to be more resistant to the cytotoxic effects of iAs, although cytotoxicity continues to be evident in the hyperplastic areas by SEM. Thus, direct mitogenesis is unlikely to be the MOA for iAs-induced bladder carcinogenesis.

Oxidative damage

Oxidative damage has been proposed as the mode of action for iAs carcinogenicity by several researchers, and therefore has drawn considerable attention (Kitchin & Conolly, 2010). However, although effects on expression of genes related to oxidative damage were observed *in vitro* (Gentry et al., 2010), the same effects have not been observed *in vivo* (Clewell et al., 2011; Suzuki et al., 2009).

A thorough examination of the *in vitro* studies that support oxidative damage as the MOA reveals that they have either used inappropriate cell types or, more commonly, were performed with arsenicals at highly cytotoxic concentrations. In many of these studies, concentrations of 100 μM or greater were used, whereas cytotoxicity occurs in the range of 0.2–5 μM (Cohen et al., 2006a; Gentry et al., 2010; Kitchin & Conolly, 2010). Cytotoxicity, with induction of cell death, clearly results in oxidative damage, but this effect is probably a consequence of cytotoxicity rather than its cause, and does not contribute to the carcinogenic process.

If the MOA for iAs cytotoxicity was oxidative damage, it should have been inhibited by antioxidants. Indeed cytotoxicity was shown to be inhibited *in vitro*, when trivalent arsenicals were co-cultured with antioxidants, such as acetylcysteine, vitamin C, and vitamin E. However, the effect did not manifest itself in *in vivo* studies (Suzuki et al., 2009). A variety of antioxidants co-administered with arsenicals to mice and rats did not inhibit the effect of the arsenicals on the urothelium, to any significant extent, particularly at the doses that cause cytotoxic effects (Cohen et al., 2006a; Suzuki et al., 2009; Wei et al., 2005). Clearly, oxidative damage, in the absence of cytotoxicity, is not the key process by which iAs causes damage in urothelial cells.

In vitro *transformation*

In vitro transformation assays have been performed utilizing urothelial cells in response to low concentrations of iAs^{III} or

MMA^{III} (Sens et al., 2004; Wnek et al., 2010). These have led to malignant transformation of the UROtsa urothelial cells, demonstrated by inoculating these cells into immunosuppressed mice. The transformation from non-malignant to malignant cells was associated with changes in Beclin-1 (associated with autophagy) (Larson et al., 2010a), various cytokines (Escudero-Lourdes et al., 2010, 2012) repression of the matrix-associated protein SPARC (Larson et al., 2010b) and other changes in gene expression (Medeiros et al., 2012). It is unclear which, if any, of these changes are the cause of the malignant transformation, or whether they are only associated with the transformation. The latter is particularly likely since they occur after, or at the time of transformation in cells that have spontaneously transformed (Sens et al., 2004; Wnek et al., 2010). This cell line transforms spontaneously to malignancy by passaging in culture over a 3-month period, the same length of time that is required for transformation by the arsenicals (Sens et al., 2004; Wnek et al., 2010). As indicated by the authors, one possibility is that the arsenical is selecting spontaneously transformed cells rather than causing the transformation itself. Furthermore, the resulting tumors are squamous cell carcinomas rather than the urothelial cell carcinomas that occur *in vivo* in humans who were exposed to high levels of iAs. The difference between *in vitro* and *in vivo* reactions may be due to the fact that the cells that were utilized in the *in vitro* assays are UROtsa cells, a human urothelial cell line that is widely used in arsenic urothelial cancer research. A major difficulty of this cell line is that the p53 gene is abnormal with expected effects on DNA repair and tumor suppression; this situation may not be relevant to carcinogenesis in humans.

Effects on DNA methylation and epigenetic changes

Effects on DNA methylation and epigenetic changes have also been suggested as the MOA of iAs carcinogenicity (Reichard et al., 2007; Rossman & Klein, 2011; Smeester et al., 2011). Most studies on this topic were done *in vitro*. In an *in vivo* study, little effect on gene expression of DNA methylation was observed in the lungs of mice that were administered 50 ppm of arsenate in the drinking water (Boellmann et al., 2010). Furthermore, some studies have reported hypomethylation rather than hypermethylation (Chen et al., 2004b). Based on the *in vivo* studies, it appears that the changes in DNA methylation that have been observed *in vitro* were the consequence of the toxic effects of arsenicals rather than the cause.

Immunosuppression

Immunosuppression has been suggested as the MOA for skin cancer associated with exposure to iAs, but not for bladder or lung cancer (NRC, 1999). There is no evidence that the urinary bladder is affected by immunosuppression, since there is no increased incidence of bladder cancer in immunosuppressed patients, and, furthermore, immunosuppressive agents do not enhance the development of bladder cancers (Cohen et al., 1991; Cohen, 2004). Even in areas of high exposure to iAs, minimal effect was observed on the immune system (Duker et al., 2005). The most common tumors seen in immunosuppressed patients are B-cell lymphomas (Cohen et al., 1991), and there is no evidence in the literature for an association between an increase in incidence of B-cell lymphomas and exposure to iAs (IARC 2004, 2012; NRC, 1999, 2001). The types of tumors that occur in immunosuppressed patients are those related to infectious diseases, principally viral, including B-cell lymphomas (Epstein Barr virus), squamous cell carcinomas (human papilloma virus, HPV), usually of the cervix, but also of the skin and other squamous epithelia, and Kaposi's sarcoma (HHV8) (Cohen et al., 1991; Cohen, 2004). Skin tumors associated with exposure to iAs are only occasionally associated with HPV (Cohen et al., 1991; Cohen & Arnold, 2011). The evidence strongly suggests that immunosuppression is not the MOA for iAs-induced bladder carcinogenesis or any other type of cancer.

Uncertainties and data gaps

There is a considerable uniformity in both qualitative and quantitative findings in various studies dealing with the urothelial response to iAs. Certain details regarding the cell transport of the thiolated arsenicals remains to be clarified, as well as details of the quantitative levels of the various arsenicals in body fluids and tissues, and the identity of the exact proteins that are affected by the trivalent arsenicals. Numerous proteins have been identified as possible targets for the reactive arsenicals. Some of these proteins, such as tubulin and keratin, are related to cell structure, whereas others, such as estrogen receptors and DNA repair enzymes, are related to various aspects of cell proliferation or its control. Although, the specific proteins that are the targets for arsenicals have not yet been elucidated, these details are not required for the understanding of the MOA but would be useful for the description of the detailed mechanisms. Such details would likely contribute to knowledge regarding organ specificity and interspecies differences, in combination with more detailed information on kinetics.

Mode of action of inorganic arsenic-induced bladder carcinogenesis: Conclusions

Considerable experimental evidence supports the MOA that is illustrated in Figure 6, including *in vivo* studies in rodents and *in vitro* experiments with a variety of cell lines, mainly urothelial and other epithelial cell lines. More information is available for the urinary bladder, from animal models and from *in vitro* investigations with corresponding cell lines than for other target organs, but evidence suggests that iAs carcinogenesis is caused by the same MOA at all of the target sites (see below).

There is a striking dose-response relationship between exposure to iAs and cytotoxicity with regenerative hyperplasia, which is non-linear and with a threshold. All studies with rodents consistently resulted in a NOEL of 1-2 ppm of iAs, regardless of the animal species (rats or mice), regardless of the form of arsenic administered (iAs^{III}, iAs^{V}, DMA^{V}), and whether in the diet or in the drinking water. The same value for a NOEL was substantiated by all investigation methods that were used (light microscopy, SEM, DNA replication labeling indices, and genomic analyses), and for all the various endpoints examined (e.g. cytotoxicity, hyperplasia

and tumors). These endpoints are observable when the generation of urinary concentration of trivalent and thiolated arsenicals are at or above the 0.1–0.2 μM range (referring to *in vitro* studies concentration data). From studies with animals it is apparent that the type of response changes with dose as well as with time. At exposures to concentrations of less than 2 ppm iAs the changes are adaptive, while at exposures to concentrations of more than 2 ppm the effect is cytotoxicity. Cytotoxicity is clearly the dominant response during the first week after administration. After the first week, the dominant response is increased proliferation, which results from the cytotoxicity. The quantitative differences in responsiveness between humans and the rodent models can be explained largely by differences in kinetics, including metabolism, cell transport, and binding to specific sulfhydryl groups of various proteins, as concluded by the 2005 SAB panel of the EPA (US EPA, 2007). A difference in organ responsiveness between species is likely due to different availabilities of sulfhydryl groups in specific targeted proteins. The consistency and reproducibility of the findings between laboratories and between different types of investigations is striking. These consistant findings strongly support the MOA of cytotoxicity and proliferation.

Inorganic arsenic-induced urothelial effects in humans

There is considerable evidence that the results of mechanistic studies with rodents, and those of targeted *in vitro* studies are relevant to humans. Qualitatively and quantitatively, the responses observed in cultured cells from mice and rats are similar to those in mice and rats *in vivo* (Cohen et al., 2006a; Drobna et al., 2005; Gentry et al., 2010; Suzuki et al., 2010; Yager et al., 2013). It is clear that concentrations greater than 5–10 μM trivalent arsenicals are lethal in tissue culture; concentrations at the range of 0.1–10 μM cause diverse cellular toxicity responses and concentrations less than 0.1–0.2 μM produce adaptive effects. Similar effects have recently been shown in human primary urothelial cells, using gene expression assessments (Yager et al., 2013).

The cytotoxicity to the urothelium of mice and rats that was described previously (Suzuki et al., 2009) was sufficient to cause cell death and regenerative proliferation, but not sufficient to cause damage to the full thickness of the urothelium, which would result in hematuria. This level of necrosis was both focal and relatively mild, and in animal studies it is detectable by SEM (Figure 5) (Cohen et al., 2007). This level of superficial cytotoxicity cannot be detected in the human urothelium using techniques that are currently available.

The effects of exposure to high concentrations of arsenic on the human urothelium were recently reported following an occupational accident in China, in which people were acutely exposed to 48.5 ± 4.3 mg/L (48.5 ppm) of iAs in well water (Xu et al., 2008). Two important findings from this acute accident support cytotoxicity being the MOA of iAs by cytotoxicity: (a) Significant levels of $TMA^{V}O$ were detected in the urine of several individuals who were exposed to the high levels of iAs, indicating that $TMA^{V}O$ can be formed in humans; and (b) approximately one-third of the exposed individuals developed hematuria, which indicates significant urothelial damage involving the full thickness of the urothelium and penetration of the basement membrane, exposing underlying capillaries. When the exposure was removed, the affected individuals recovered from the iAs-induced toxicity and the hematuria disappeared, indicating tissue repair. Such tissue repair is in line with the reversibility that has been observed with various other stimuli which produced urothelial toxicity and hematuria in rats (Cohen et al., 2007; Fukushima et al., 1981).

In individuals exposed to elevated levels of iAs in drinking water and/or food, that are significantly lower than the levels in the occupational accident described above, the cytotoxicity to the urothelium would be expected to be superficial, similar to the effect observed in the rodent models, which does not result in hematuria. Support for this hypothesis comes, for example, from a study showing an increase in the number of exfoliated cells found in the urine of individuals in Chile that were exposed to high levels of iAs in the drinking water [up to approximately 530 μg/L (0.53 ppm)] (Moore et al., 2002), as well as from PML patients treated with arsenic trioxide (0.150 mg/kg body weight/day, intravenously) (Wedel et al., 2013). The observation of exfoliated cells in urine suggests that the iAs exposure in those cases was associated with superficial cytotoxicity that was insufficient to cause hematuria.

Urinary arsenic and its trivalent metabolites in humans

For the assessment of the dose-response relationship of iAs and its trivalent metabolites in humans, it is important to compare the findings in humans with those of animal studies.

Animal studies provide evidence that exposure to iAs doses that result in trivalent urinary metabolites at concentrations lower than 0.1 μM do not cause adverse effects in the bladder, while trivalent metabolite concentrations greater than 0.1 μM (usually greater than 0.2–0.5 μM) are associated with urothelial cytotoxicity. High levels of trivalent metabolites of arsenic in urine, together with prolonged exposure, are expected to be associated with carcinogenesis. For example, in studies with DMA^{V} in which rats were fed DMA^{V} at a concentration of 100 mg/kg feed, a dose that was found to be carcinogenic, the concentration of DMA^{III} in the urine was 1.38 μM on day 8 of the exposure (Cohen et al., 2006a; Lu et al., 2003).

Only a few epidemiological studies have measured iAs and its trivalent metabolites (i.e. $As^{III} + MMA^{III} + DMA^{III}$) in human urine (Tables 1 and 2). Despite this limited data set and the uncertainty associated with measuring concentrations of arsenicals in the urine (see the section titled "Definition of the Arsenic Species Analyzed"), the results provide prospective on the levels of trivalent arsenicals in urine at different exposure levels and are consistent with the proposed MOA for iAs bladder carcinogenicity involving a threshold. That is, only drinking water concentrations of iAs greater than 100 μg/L are associated with cytotoxic concentrations of trivalent metabolites in the urine. As discussed earlier, increases in bladder cancer have been reliably observed in epidemiological studies only when drinking water concentrations exceed 100 μg/L. Moreover, lower concentrations of

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Table 1. Concentrations of inorganic arsenic and its trivalent metabolites in urine of populations or individuals following ingestion of less than 100 μg arsenic/L drinking water.

Study Area	Exposure Group	n	DW Conc. (μg/L)	As^{III} (μg/L)	As^{V} (μg/L)	MMA^{III} (μg/L)	MMA^{V} (μg/L)	DMA^{III} (μg/L)	DMA^{V} (μg/L)	Total As (μg/L)	Total Trivalents (μg/L)*	Total Trivalents (μM)*	% Trivalents	Reference
Zimapán and Lagunera Mexico	Arithmetic Mean	258	42.9	4.2	1.4	0.4	5	4.7	22.5	41.2	9.3	0.12	23	Del Razo et al., 2011
	GeometricMean	258	24.4	2.1	1.5	0.1	1.1	0.9	2.4	24.7	3.1	0.04	13	
	Low	1†	3.1	0.1	ND	ND	0.1	ND	0.1	2.3	0.1	0.00	4	
Zimapán, State of Hidalgo, Mexico	Subject 1	1	30–1100‡	8.1	4.5	ND	7.1	ND	18.6	41.8	8.1	0.11	19	Del Razo et al., 2001
West Bengal, India	Group A	105	33			3		8		50.8	11	0.15	22	Mandal et al., 2001
West Bengal, India	Group A	8	29	9.27	8.58	6.49	8.63	6.31	25.8	66.5	22.07	0.29	33	Mandal et al., 2004
	Group B	8	55	11.2	8.94	7.44	10.3	16.8	31.5	87.9	35.44	0.47	40	
Zimapán, State of Hidalgo, Mexico	Control	28	1.6	1.6¶	3.6¶	2.2¶	0.6¶	7.9¶	7.4¶	33.3¶	11.7	0.16	35	Valenzuela et al., 2005
Romania	Group A	14	2.8	NM	NM	ND	NM	NM	NM	NM	NM	NM	NM	Aposhian et al., 2000
	Group B	14	29	NM	NM	4.8§	NM	NM	NM	NM	NM	NM	NM	
	Group C	14	84	NM	NM	5.7§	NM	NM	NM	NM	NM	NM	NM	

*Calculated based on reported study results.
†Reflects the lowest drinking water measurement and arsenic urine measurement (not necessarily for the same individual because individual data not provided).
‡Drinking water level for each individual was not specified, but general As concentrations in the area range from 30–1100 μg/L. Consistent with other studies, we assumed that if total arsenic in urine was less than 100 μg/L then drinking water concentrations were also less than 100 μg As/L (and conversely if total arsenic in urine was greater than 100 μg/L then drinking water concentration were assumed to be greater than 100 μg As/L).
¶Study reported urine results in μg As/g creatinine. For the purposes of this table we assumed that typical adult urine contains approximately 1 g creatinine/L of urine (http://www.mayomedicallaboratories.com/test-catalog/Clinical+and+Interpretive/90480).
§Mean of samples where MMA^{III} was detected.
DW = Drinking water; ND = Not detected; NM = Not measured.

Table 2. Concentrations of inorganic arsenic and its trivalent metabolites in urine of population following ingestion of greater than 100 µg arsenic/L drinking water.

Study Area	Exposure Group	n	DW Conc. (µg/L)	As^{III} (µg/L)	As^{V} (µg/L)	MMA^{III} (µg/L)	MMA^{V} (µg/L)	DMA^{III} (µg/L)	DMA^{V} (µg/L)	Total As (µg/L)	Total Trivalents (µg/L)*	Total Trivalents (µM)*	% Trivalents	Reference
Zimapán and Lagunera regions Mexico	High	1†	215	37.2	32.9	2.4	36.2	64.8	162.6	233.7	104.4	1.39	45	Del Razo et al., 2011
Zimapán, State of Hidalgo, Mexico	Subject 2	1	30–1100‡	11.2	12.2	1.1	11.2	18.3	52.7	122.2	30.6	0.41	25	Del Razo et al., 2001
	Subject 3	1	30–1100‡	25.2	16.8	2.3	37.7	18.2	40.1	169.3	45.7	0.61	27	
	Subject 4	1	30–1100‡	14.7	10.1	1.2	19.3	5.7	90.5	147.4	21.6	0.29	15	
	Subject 5	1	30–1100‡	59.4	72.1	5.2	119.8	59.8	408.2	734.6	124.4	1.66	17	
	Subject 6	1	30–1100‡	104.2	122.8	12.3	276.7	114.2	467.2	1211.1	230.7	3.08	19	
West Bengal, India	Group B	120	148			6		18.6		195	24.6	0.33	13	Mandal et al., 2001
	Group C	156	210			10		42		242	52	0.69	21	
	Group D	47	248			30		63.8		1487	93.8	1.25	6	
West Bengal, India	Group C	12	130	19.9	17.7	9.37	14.9	23.1	80.8	167	52.37	0.70	31	Mandal et al., 2004
	Group D	13	163	22.9	19.3	11.9	28.5	32.3	171	287	67.1	0.90	23	
Zimapán, State of Hidalgo, Mexico	Exposed w/o lesions-mean	21	117	6.9¶	6.1¶	4.8¶	1.8¶	48.1¶	15.9¶	116¶	59.8	0.80	52	Valenzuela et al., 2005
	Exposed w/ lesions-mean	55	115	6.3¶	8.2¶	7.5¶	2.0¶	51.9¶	19.8¶	121.2¶	65.7	0.90	54	
Romania	Group D	16	161	NM	NM	6.9§	NM	NM	NM	NM	NM	NM	NM	Aposhian et al., 2000

*Calculated based on reported study results.

†Reflects the highest drinking water measurement and arsenic urine measurement (not necessarily for the same individual because individual data not provided).

‡Drinking water level for each individual was not specified, but general As concentrations in the area range from 30–1100 µg/L. Consistent with other studies, we assumed that if total arsenic in urine was less than 100 µg/L then drinking water concentrations were also less than 100 µg As/L (and, conversely, if total arsenic in urine was greater than 100 µg/L then drinking water concentration were assumed to be greater than 100 µg As/L).

¶Study reported urine results in µg As/g creatinine. For the purposes of this table we assumed that typical adult urine contains 1 g creatinine/L of urine (http://www.mayomedicallaboratories.com/test-catalog/Clinical+and+Interpretive/90480).

§Mean of samples where MMA^{III} was detected.

DW = Drinking water; NM = Not measured.

arsenic in drinking water are not associated with sufficient urinary levels of trivalent arsenicals to cause significant bladder cell cytotoxicity. Specifically, concentrations of less than 100 μg/L of iAs in drinking water, which are below the epidemiologically-based carcinogenic threshold, generally result in concentrations of less than 0.2 μM of trivalent iAs, its trivalent metabolites, i.e. cytotoxic metabolites ($iAs^{III} + MMA^{III} + DMA^{III}$), and the small amounts of thiolated arsenicals in the urine.

Tables 1 and 2 present a listing of concentrations of trivalent iAs and its trivalent metabolites in urine from studies that measured urine concentrations. When populations were exposed to concentrations of iAs in drinking water less than 100 μg/L (Table 1), urinary levels of the trivalent arsenic species ranged from 0.001 to 0.16 μM, levels that are insufficient to produce urothelial cytotoxicity [total μM of trivalent arsenic compounds calculated based on individual trivalent arsenic urinary measurements (i.e. $iAs^{III} + MMA^{III} + DMA^{III}$)]. This range excludes the results from Mandal et al. (2004), which were questioned by Hansen et al. (2004) who concluded that the analytical method used by Mandal et al. [originally published in Reay & Asher (1977)] for trivalent metabolites was flawed as it measured DMA^{V} and not DMA^{III}. This may explain the difference between the results of Mandal et al. (2004) and those of the other researchers. In humans ingesting drinking water with iAs concentrations greater than 100 μg/L, the range of urinary levels of inorganic arsenic and its trivalent metabolites is 0.33 to 3.08 μM (Table 2), which, in animal models, was shown to be associated with bladder cytotoxicity followed by urothelial proliferation that can lead to cancer after prolonged exposure.

For example, Del Razo et al. (2011) showed that exposure of a large population ($n = 258$) to an average concentration of 42.9 μg/L of iAs in drinking water, resulted in average urinary concentration of 0.12 μM trivalent arsenic species, a level that is not associated with bladder cytotoxicity or carcinogenicity in animals. In contrast, in the study of Valenzuela et al., (2005), the mean concentration of iAs in drinking water of the exposed groups was 115–117 μg/L. These drinking water levels were associated with average urinary concentrations of 0.8–0.9 μM of trivalent arsenic species. [Note: Trivalent arsenic concentrations in urine reported in μg/g creatinine were converted to μg/L, assuming typical adult urine contains 1 g creatinine per liter of urine. Total μM of trivalent arsenic compounds calculated based on individual trivalent arsenic urinary measurements (i.e. $iAs^{III} + MMA^{III} + DMA^{III}$)]. Valenzuela et al. (2005) did not evaluate bladder cancer as a health endpoint, but these levels are expected to be associated with bladder cytotoxicity. These findings are consistent with the extensive epidemiology literature that shows that exposure to iAs in drinking water above the cancer NOEL results in urinary trivalent metabolites at levels which are associated with cytotoxicity.

In the US, the National Health and Nutrition Examination Survey (NHANES) has recently examined total arsenic as well as levels of seven arsenic compounds, including arsenobetaine, MMA and DMA, in the urine of US populations ($n \approx 2800$). The highest levels of total As in urine were found in the most recent survey (2009–2010) (CDC 2012). In this data set, the median concentration of the total arsenic compounds found was 8.15 μg/L (including 0.94 μg/L of arsenobetaine). This study did not distinguish between methylated pentavalent and trivalent forms of arsenic, but the levels of iAs and its trivalent arsenic metabolites ($iAs^{III} + MMA^{III} + DMA^{III}$) in the population can be estimated based on the relative proportions of the metabolites that were found in other studies. According to Del Razo et al. (2011), the portion of trivalent arsenic compounds in urine is about 23%. Applying this value to the total concentration of arsenic in the urine of the US population measured by NHANES (minus the known arsenobetaine levels) of the US population measured by NHANES, urinary concentrations of trivalent metabolites would be estimated to be only 0.02 μM at the 50th percentile and 0.11 μM at the 95th percentile (Table 3). These concentrations are below the levels that are associated with bladder cytotoxicity, and therefore would not be a concern for increased risk of bladder cancer.

Table 3. Levels of arsenic in the urine of US population (Data from NHANES 2009–2010*).

Form of Arsenic	50th Percentile	95th Percentile
Total Arsenic (Urine)	8.15 μg/L	85.6 μg/L
Arsenobetaine	1.59 μg/L	50.4 μg/L
Total Arsenic (Urine) MINUS Arsenobetaine	6.56 μg/L	35.2 μg/L
Estimated Concentration of Trivalent Metabolites†	1.57 μg/L	8.10 μg/L
Estimated Concentration of Trivalent Metabolites	0.02 μM	0.11 μM

*Data available at http://www.cdc.gov/exposurereport/pdf/FourthReport_UpdatedTables_Sep2012.pdf 314p.

†Assuming that 24% of arsenic in urine is in trivalent form, based on average levels reported in Del Razo et al., (2011); calculated as $[(As^{III} + MMA^{III} + DMA^{III}/(As^{III} + MMA^{III} + DMA^{III} + As^{V} + MMA^{V} + DMA^{V})]$.

Quantitative information regarding human exposures that present a concern for bladder cytotoxicity and carcinogenesis may be gleaned from the data from epidemiological studies, together with the data from animal studies, thus enabling quantitative comparisons across species and bridging between human exposures and information on the MOA from animal studies. The analysis presented in Table 3 indicates that exposures of humans to iAs in drinking water at concentrations that are less than 100 μg/L do not pose a risk of cancer, because the concentrations of trivalent arsenic in urine resulting of such exposures are insufficient to cause significant cytotoxicity. Rodents are much less sensitive to ingested iAs than humans, due, primarily, to toxicokinetic differences between species. Only oral exposures to concentrations greater than 1000 μg/L in the drinking water or 1000 mg/kg of diet, produce cytotoxic levels of trivalent arsenic compounds in the urine of rodents. In 2008, El-Masri and Kenyon published the first human PBPK model that allowed the prediction of trivalent metabolites in urine (El-Masri & Kenyon, 2008). This model predicts the concentrations of trivalent arsenicals in the urine reasonably well, and is supported by studies in human populations (Aposhian et al., 2000; Le et al., 2000; Valenzuela et al., 2005).

A PBPK model that predicts the concentrations of trivalent arsenic species in mouse organs and urine has been under

development by Clewell et al. (2007) and Kenyon et al. (2008a). These developments are important steps for the understanding of the quantitative differences between the metabolism of iAs in animals and in humans. Ultimately, when these differences in metabolism are quantified, this PK information will be combined with that from the research on MOA that is being conducted *in vitro* and *in vivo*, to build an integrated biologically-based dose response model. In any case, the available evidence supports well the existence of a threshold for iAs, and the finding that exposure to iAs in drinking water containing arsenic less than 100 µg/L does not produce urinary metabolites at concentrations that are sufficient to cause cytotoxicity, and which might increase the risk of cancer in the exposed populations.

Ideally, to provide a more robust understanding of how much ingested iAs is required to produce cytotoxic urinary concentrations of trivalent arsenicals, a study evaluating urinary arsenic levels of trivalent iAs and its trivalent metabolites in individuals consuming known amounts of iAs in the drinking water and diet would be useful. Such a study could also shed light on human variability, particularly if urine samples were collected from each individual for several consecutive days. Such an experiment has not been published and would be logistically difficult, but would provide the kinds of data necessary for a more accurate estimate of the threshold amount of ingested iAs that could generate a cytotoxic concentration of trivalent arsenicals in the urine.

Effects of inorganic arsenic on lung and skin

Effects on the lung

Lung cancer is induced in individuals that had been exposed to high levels of iAs, either by inhalation in occupational settings, primarily in smelting industries and in coal burning areas where coal with high contents of iAs is utilized (e.g. China), or by oral ingestion in the drinking water (Bencko et al., 2009; Celik et al., 2008; IARC 2004, 2012; NRC, 1999, 2001). There are multiple studies associating iAs in drinking water with lung cancer in humans (e.g. Chen et al., 1988a,b, 2004a; Guo, 2004; Marshall et al., 2007). There are few studies of iAs in drinking water and lung cancer in animals (Tokar et al., 2010b). Effects of exposure to iAs by inhalation have received even less attention.

Evidence for an effect on lung in an animal model was observed by Waalkes et al., (2003) following transplacental exposure by administration of 42.5 ppm, or 85 ppm, of sodium arsenite in drinking water, from day 8 to 18 of gestation (Tokar et al., 2010a,b; Waalkes et al. 2003). The offspring were observed throughout their lifetime, with no further administration of iAs. An increased incidence of lung tumors was found in the offspring of the highest dose group (85 ppm). In a follow up study, involving similar transplacental exposure and continued exposure of the offspring through the drinking water for the duration of their lifetime, a similar increase in lung tumors was observed in the highest dose group (Tokar et al., 2010a). However, these studies have several limitations which make extrapolation of the results to humans difficult:

(a) The response has been limited to strains of mice that are characterized by spontaneous high incidences of lung tumors (e.g. strain A) (Nikitin et al., 2004; Tokar et al., 2010a,b). Furthermore, Cytochrome P450 2f2, a major metabolizing enzyme in the mouse lung, does not exist in humans (Cruzan et al., 2012). This enzyme appears to be a significant metabolizing enzyme for a variety of xenobiotics in mice, although its effect on iAs is unknown. It appears that Cytochrome P450 2f2 contributes, at least to some extent, to the higher susceptibility of strains of mice that have, higher spontaneous incidences of tumors in lungs than other strains (Cruzan et al., 2009, 2012);
(b) An increase in the size of the observed tumors in mice treated with iAs compared to controls and in the number of tumors per mouse in most studies was reported, but usually not an increase in the incidence (i.e. the percentage of mice affected) (Cui et al., 2006; Hayashi et al., 1998);
(c) The increased incidence of lung tumors in some of these studies was relatively small, and no information was provided regarding the historical background incidences of lung tumors in the strains in these laboratories;
(d) The mouse is not a representative model for human lung carcinogenesis (Boorman & Eustis, 1990; Nikitin et al., 2004; Travis et al., 2004). First, the bronchiolar alveolar structure of the mouse is different from that of humans, so that the aerodynamics and the exposure to substances in the airways are different (Boorman & Eustis, 1990; Plopper et al., 1980; Travis et al., 2004). Thus the relationship between air particulates and dose delivered to different pulmonary regions differ between these species. Second, lung tumors in mice are of glandular origin, arising at the periphery of the lung, and progressing through stages of hyperplasia, adenoma, and adenocarcinoma (Boorman & Eustis, 1990; Nikitin et al., 2004), whereas, human lung tumors have a different pathogenesis with no pre-cancerous adenoma (Travis et al., 2004). In humans, tumors arise either at the periphery (bronchiolo-alveolar adenocarcinomas) or centrally, as the more typical adenocarcinoma. The tumors that arise centrally are derived from the bronchial epithelium, similar to the squamous cell carcinoma and the small and large cell undifferentiated carcinomas. The most common types of tumors related to cigarette smoking and to exposure to iAs are squamous cell and small or large cell undifferentiated cell carcinomas (Chen et al., 2010b; Travis et al., 2004).

Interestingly, direct administration of iAs to mice by intratracheal administration had little effect on the lower respiratory tract, whereas, similar studies in mice with chromium (VI), which like arsenic is an established human carcinogen by inhalation (ATSDR, 2012c), showed an increase in lung tumors, and arsenate co-administered with chromium appeared to enhance the effect of chromium (Tajima et al., 2010).

Recently, Chilakapati et al. (2010) demonstrated that human bronchial epithelial cells in culture are susceptible to the cytotoxic effects of trivalent, oxygen-containing arsenicals and to thiolated arsenicals, similar to urothelial cells, and at similar concentrations. The bronchial epithelial cells were an established cell line, with known abnormality of p53.

Table 4. Comparison of values obtained for the LC_{50} of various metabolites of arsenic to human bronchial epithelial (HBE) cells *in vitro*, with LC_{50} values from previous studies of the same compounds to human urothelial cells (ITI)*.

	iAs^{III}	iAs^{V}	MMA^{III}	MMA^{V}	DMA^{III}	DMA^{V}	$DMMTA^{V}$
1T1†	4.8	31.3	1.0	1700	0.8	500	–‡
1T1¶	8.3	34.6	0.9	2700	1.0	230	1.4
HBE cells§	5.8	46.5	1.0	6100	1.4	960	5.5

*LC_{50} values are expressed as μM.
†Cohen et al., 2002
‡Not performed.
¶Suzuki et al., 2010
§Dodmane et al., 2013a

Alterations in p53 signaling and in cell-cycle-related genes were observed in a gene expression analysis that was performed by Chilakapati et al. (2010). However, the use of a cell line with an abnormal p53 gene makes the interpretation of these findings difficult. The observed changes in genes are related to oxidative stress, but the implications of the finding for the *in vivo* situation are unclear. Similar observations, with the same established cell line, were reported by Clancy et al. (2012). As explained earlier, it is likely that the oxidative stress is secondary to the cytotoxicity. Although these studies were performed utilizing established cell lines, Arnold et al. (2011) and Dodmane et al. (2013a) came to the same conclusions regarding cytotoxicity when evaluating the IC_{50} for primary human bronchial epithelial cells. IC_{50} values reported by Dodmane et al. (2013a) were as follows: iAs^{III} - 5.3 μM, MMA^{III} - 1.9 μM, DMA^{III} - 1.4 μM, and dimethylmonothioarsinic acid ($DMMTA^{V}$) - 5.5 μM. These values are similar to the IC_{50} values for human urothelial cells (Table 4) and for human keratinocytes (Drobna et al., 2005; Klimecki et al., 1997; Suzuki et al., 2010). In addition, gene expression analysis following exposure to iAs^{III}, MMA^{III}, or DMA^{III} showed similar pathway alterations in human bronchial epithelial cells (primary or established cell line), urothelial cells and skin keratinocytes (Chilakapati et al., 2010; Clancy et al., 2012; Dodmane et al., 2013a). Taken together, these various *in vitro* investigations support the hypothesis that cytotoxicity is the key event in all three tissues in their response to trivalent arsenicals, with a similar dose response and similar cellular response as reflected by gene expression analyses.

Oxygen-containing pentavalent arsenicals caused toxic effects in the human bronchial epithelial cells only in the millimolar concentration range, similar to the concentrations with human urothelial cells and human keratinocytes. Bartel et al. (2011) observed cytotoxicity of arsenic metabolites, including thiolated species, in human lung cells, but their study involved the use of a human lung adenocarcinoma cell line, a malignant cell line. A similar cytotoxicity study has not been performed in lung cells from mice or rats, partly due to the unavailability of appropriate cells, especially primary cells, and because of the questionable relevance of such a study and its results, given the lack of bronchogenic-derived tumors in either of these species. In spite of all these reservations, the available *in vitro* studies that use human cells support a MOA for the lungs that involves cytotoxicity followed by regeneration.

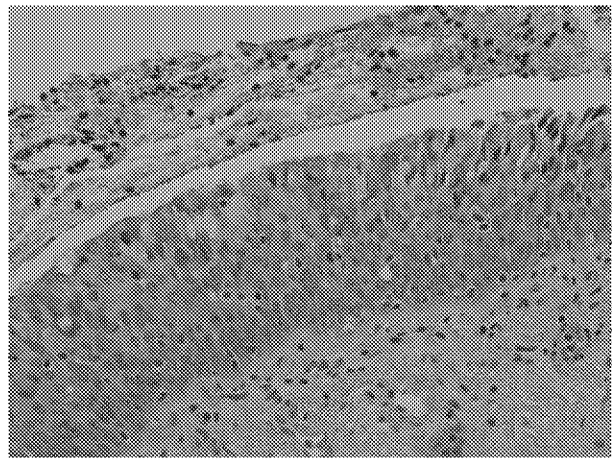

Figure 8. Chronic bronchitis in cigarette smoker showing chronic inflammation and mucosal hyperplasia. A layer of mucous is present in the lumen. Similar changes can be produced not only by arsenic exposure, but also by cigarette smoking, allergy, other environmental exposures, or infections.

There are a limited number of studies in humans regarding preneoplastic changes in the respiratory tract in response to exposure to arsenic. The existing studies suggest that high exposures to iAs in drinking water (Mazumder, 2007; Mazumder et al., 2000; Milton & Rahman, 2002; Parvez et al., 2008, 2010; von Ehrenstein et al., 2005) and possibly in air (ATSDR, 2007) are related to increased rates of bronchitis or bronchiectasis. The pathogenesis of bronchitis involves cytotoxicity and regeneration, which can be a precursor to hyperplasia, metaplasia, dysplasia, and carcinoma (Figure 8) (Travis et al., 2004). Many of these studies have limitations regarding ascertainment of respiratory changes and specific diagnoses. Like the urothelium, the bronchial epithelium is a slowly proliferating tissue. With bronchitis, the bronchial epithelium, like the urothelium, becomes considerably more active mitotically.

In summary, there is a growing body of evidence that the MOA for the formation of lung tumors due to exposure to iAs is the same as for tumors of the urinary bladder. The presence of high levels of surfactant protein that lines the lung epithelium (Kishore et al., 2006) may play a role in molecular interactions between iAs and the lung. Surfactant proteins, which are rich in cysteines, may provide high concentrations of sulfhydryl groups that interact with trivalent arsenicals, resulting in cytotoxicity of the bronchial epithelium. Unlike the urinary bladder, the MOA for lung tumors cannot be demonstrated in animals because of the lack of an appropriate animal model. Nevertheless, there is sufficient evidence to indicate that the MOA is that of cytotoxicity followed by regeneration. Similar levels of trivalent and thiolated arsenicals cause the same cytotoxicity to human bronchial epithelial cells in culture as observed for urothelial cells and keratinocytes (Chilakapati et al., 2010; Dodmane et al., 2013a). Furthermore, the cellular gene expression changes are similar in all three cell types. Studies of precursor changes in certain lung diseases, such as chronic bronchitis, which has been associated with iAs, are also consistent with toxicity and regenerative proliferation as a MOA (Mazumder et al., 2000;

Mazumder, 2007; Milton & Rahman, 2002; Parvez et al., 2008, 2010; von Ehrenstein et al., 2005).

Effects on the skin

Skin cancer, with associated skin changes, was the first carcinogenic effect identified following chronic exposure to arsenic (Cullen, 2008; Hughes et al., 2011; Schuhmacher-Wolz et al., 2009). This association was first noticed in patients receiving arsenic-containing pharmaceuticals; association with high levels of iAs in drinking water was discovered several decades later (Cullen, 2008). Skin has received considerable attention in attempts to develop animal models (Tokar et al., 2010a,b; Yu et al., 2006). The studies have included skin painting as well as oral administration, following the tradition of skin carcinogenesis experiments that have been performed over the past century for a number of other agents (Rossman et al., 2004), These studies were done with various, specially developed strains of mice, such as Tg.AC transgenic mice, in which multiple copies of the H-ras gene are incorporated into the genome (Germolec et al., 1998; Waalkes et al., 2008). However, no adequate animal model for the development of skin cancer has yet been found. Administration of various arsenicals did not increase the incidence of skin tumors in the p53 heterozygous mice (Salim et al., 2003) or in K6/ODC mouse (Chen et al., 2008). The latter strain is thought to be equivalent to a pre-initiated mouse, which is very sensitive to the development of skin tumors following dermal exposure (Chen et al., 2008). Extensive investigations, under artificial circumstances, utilized the traditional initiation-promotion model that was originally developed by Berenblum & Shubik (1947), and was further developed by Boutwell (1964) and Slaga (1983a,b) and their associates. Using this mouse skin model, there is no clear evidence that iAs acts as either an ''initiator'' or as a ''promoter'' (Rossman et al., 2004). Interestingly, in the transplacental model that was developed by Waalkes et al. (2003), tumors were observed in multiple organs, but not on skin following transplacental exposure to iAs or when transplacental exposure was followed by exposure of the offspring to 12-*O*-tetradecanoyl phorbol-13-acetate (TPA), a well-known promoter of skin tumors in mice (Waalkes et al., 2004).

In vitro studies of human tissues provide considerable evidence that there is a similar MOA for skin carcinogenesis, in response to exposure to iAs, similarly to the urothelium and the lung. For example, *in vitro* studies of the response of human keratinocytes to arsenicals gave similar evidence for cytotoxicity as seen with urothelial and bronchial epithelial cells, with comparable LC_{50}'s for the trivalent arsenicals (Bae et al., 2001; Chen et al., 2005; Liao et al., 2011; Mudipalli et al., 2005; Trouba & Germolec, 2004; Vega et al., 2001). The effect of thiolated arsenicals in keratinocyte cultures, are expected to be similar to those observed on the urothelium (Suzuki et al., 2009) and bronchial epithelial cells (Chilakapati et al., 2010), although we are not aware that such an evaluation has been performed.

When iAs is co-administered simultaneously with UV radiation there is an indication for a co-carcinogenic effect (Klein et al., 2007; Rossman et al., 2004; Yu et al., 2006).

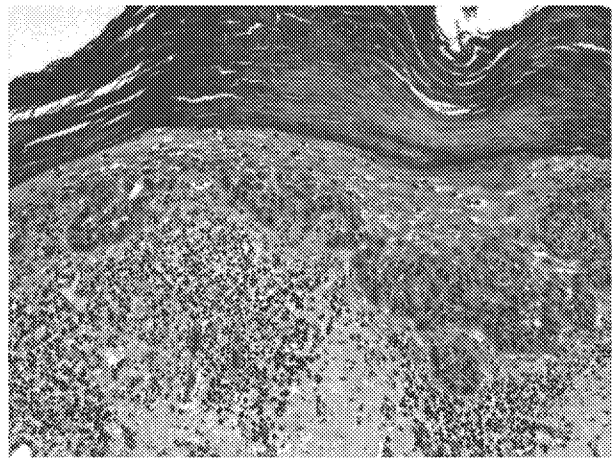

Figure 9. Skin showing chronic inflammation, epidermal hyperplasia and hyperkeratosis. Such changes can occur secondary to high arsenic exposure or excessive sun exposure.

This may be relevant to humans, but only to a limited extent, since changes in the skin that were induced by iAs occur both on sun-exposed and on unexposed skin (Schuhmacker-Wolz et al., 2009). The major characteristics of arseniasis are hyperpigmentation, or less commonly, hypopigmentation of the skin, in addition to hyperkeratosis associated with chronic inflammation and epidermal hyperplasia, predominantly on the palms and soles (Figure 9) (Schuhmacher-Wolz et al., 2009; and in Hughes et al., 2011). Hypopigmentation reflects a decrease in the amount of melanin in the epidermis, and since melanin is the skin's major defense against absorption of free radicals that are generated by exposure to UV radiation, fair-skinned individuals, who have naturally decreased amounts of skin melanin are at a considerably greater risk of developing skin cancer than heavily pigmented individuals, with albinos being the most sensitive to this effect (Cohen & Arnold, 2011). iAs decreases the amount of melanin in the skin, thus increasing the risk for damage to the DNA by UV radiation, at least at sites that are heavily exposed to sunlight (Melkonian et al., 2011). However, arsenicals can be involved also with hyperpigmentation (Schuhmacher-Wolz et al., 2009).

Qin et al. (2012) suggested that iAs induced an effect on DNA repair in the skin, specifically for UV-induced damage, but possibly affecting also repair of bulky chemical DNA adducts such as those occurring with exposure to polycyclic aromatic hydrocarbons (Shen et al., 2009). These studies were done *in vitro* and used cells that already had p53 defects, with associated defects in DNA repair. The effects on DNA repair occurred at concentrations that are high and probably not attainable in humans. Furthermore, these observations do not address the occurrence of arsenic-induced lesions in skin which is not exposed to sun as well as lesions occurring at sites that are sun exposed.

Cigarette smokers are more susceptible than non-smokers to arsenic-induced skin changes, including neoplasms, similar to the synergy in the bladder and lung (Chen et al., 2006; Lindberg et al., 2010; Melkonian et al., 2011; Yu et al., 2006). A more reasonable explanation than effects on DNA repair, suggested by Lindberg et al. (2010), is a possible effect of smoking on arsenic methylation.

Hyperkeratosis (Figure 9) that is associated with arsenic exposure suggests that the initial changes in the epidermis following exposure to iAs are superficial, similar to the effect on the rodent urothelium. An inflammatory response would be expected only when the toxicity involves the full thickness of the epidermis. Chronic inflammation is often associated with hyperkeratosis, but there have been reports of individuals exposed to high concentrations of iAs with hyperkeratosis and hypopigmentation with no chronic inflammation, or with the inflammation occurring at a later stage (Schwartz, 1996).

Arsenicals concentrate in the skin probably because of the extremely high amount of sulfhydryl groups in epidermal proteins, particularly in keratins (Cullen, 2008; Hughes et al., 2011). It is unclear what the effect of the interaction with these keratins is, since they are primarily structural proteins, but accumulation of sufficient amounts of arsenic in the skin cells is expected to cause a toxic response that would lead to cell death.

Arseniasis and ultimately skin cancer develop only after exposure to high concentrations of iAs over a long period of time, usually decades (Chen et al., 2009; Hughes et al., 2011; Schuhmacher-Wolz et al., 2009; Yu et al., 2006). The epidemiology for the benign, precancerous skin changes is similar to that for skin cancer and iAs. Increases in incidence are typically observed only after the concentrations in the drinking water to which the population is exposed, exceed 100 ppb (e.g. Lindberg et al., 2008; Tondel et al., 1999; Yang et al, 2002), although some studies have found skin lesions in populations exposed to lower concentrations (Ahsan et al., 2006; Argos et al., 2011). The conflicting reports regarding the association between exposure and skin arseniasis, in contrast to the consistent findings of skin cancer only at exposures to higher iAs concentrations, could be due to the greater variablility of the clinical and pathologic appearance of the non-neoplastic skin changes, and possibly to misclassification of the lesions. Only some of the skin changes are actually preneoplastic, e.g. actinic keratosis in contrast to the more seborrheic keratosis-like change. Skin changes, which are the toxic manifestations of exposure to iAs, are reversible before they convert to carcinoma (Seow et al., 2012). However, it is not clear from these reports which of the various benign skin changes associated with iAs are reversible and which are not.

In summary, accumulating evidence from epidemiological investigations, histopathology of precancerous changes, and *in vitro* investigations, show that similar to urinary bladder and lung, the MoA of iAs for skin is cytotoxicity to the skin epidermis (hyperkeratosis) and regenerative proliferation (acanthosis, epidermal hyperplasia), leading to cancer in individuals exposed to high levels of iAs in the drinking water.

Nonlinearity and threshold: cancer and non-cancer

The dose-response relationship for iAs-induced effects in the various tissues is clearly non-linear, based on results from both *in vitro* and *in vivo* investigations. The only MOA that might have caused a linear, non-threshold dose-response relationship for carcinogenicity is direct reaction with DNA; however, iAs is not DNA-reactive as was shown by Nesnow et al. (2002).

All of the mechanisms which were proposed for the carcinogenesis of iAs lead to a non-linear process with a threshold. All of the proposed modes of action involve precursor key events that are non-cancerous (i.e. superficial cytotoxicity of the urothelium, bronchitis of the lungs and arseniasis of the skin, see Figure 7), which lead to cell death and consequent increased cell proliferation (Boobis et al., 2006; Chen, 2004a; Cohen & Arnold, 2011; Cohen & Ellwein, 1991; Cohen et al., 2004; Meek et al., 2003). A similar process probably occurs in the other tissues in which high iAs exposures are associated with cancer, such as liver and kidney (NRC, 1999, 2001). High iAs exposures do not cause cancer directly, but produce toxic effects in epithelial tissues that result in cell death with consequent regenerative cell proliferation. If continued for a prolonged period of time, these effects increase the risk of cancer (Cohen & Ellwein, 1990, 1991; Moolgavkar & Knudson, 1981).

Cytotoxicity and regenerative proliferation is a frequently occurring MOA for non-DNA reactive urinary bladder carcinogens, whether cytotoxicity is produced by formation of urinary solids (e.g. melamine) or due to chemical cytotoxicity secondary to concentration and urinary excretion of a cytotoxic metabolite (e.g. tributyl phosphate) (Cohen, 1998; Meek et al., 2003). Regardless of the inciting agent, there is a threshold for cytotoxicity and consequently a threshold for carcinogenicity.

For iAs, there is compelling evidence that a threshold is involved for the precursor changes, and consequently for the carcinogenic effect. This was best demonstrated for rat bladder cancer induced by DMA^V, which involves generation of DMA^{III} that is one of the reactive metabolites produced by iAs. The cytotoxicity and regeneration that were induced in the urothelium by these reactive metabolites were reversible when the cytotoxic agent was removed (Arnold et al., 1999; Cohen, 1998; Cohen et al., 2006a; Fukushima et al., 1981).

There are several sources of evidence demonstrating a threshold for iAs. Epidemiology strongly supports iAs-induced cancers occurring in humans exposed to iAs in drinking water only at concentrations greater than 100–150 ppb (μg/L). Animal models are strikingly consistent, demonstrating a NOEL of approximately 1–2 ppm of iAs in the drinking water or in the diet, for any effects on the urothelium, using any investigation method, including transcription array expression analysis. Human exposure to iAs concentrations of 100-150 μg/L in drinking water results in urinary concentrations that are comparable to concentrations resulting from rodent exposure to 1–2 mg/L in drinking water or in diet. No adequate animal model is available for arsenic-induced cancer of the skin or lung. However, *in vitro* studies with arsenicals, utilizing a variety of human cell types, including urothelial cells, bronchial epithelial cells and keratinocytes, have shown a no observed adverse effect level of approximately 0.1–0.2 μM (Gentry et al., 2010).

The nonlinear dose-response relationship of the metabolism of iAs, which is demonstrated by extensive *in vitro* and *in vivo* investigations in animal models, as well as studies in humans, can be explained on the basis of the enzymes involved combined with cell transport and kinetics. The most apparent indication of the nonlinearity of metabolism in humans is that TMA^VO is found only when the exposure to

iAs is extremely high, unlike rodents in which TMA^VO is found following exposure to much lower iAs doses.

iAs-induced cancer of the bladder, lung, and skin begin as non-cancerous lesions, formed as a result of cytotoxicity with consequent regenerative proliferation induced by iAs and its metabolites (Figure 7). Cytotoxicity with consequent regenerative proliferation is often associated with inflammation. These pre-cancerous lesions are reversible when exposure is discontinued. Based on the existing information, it is reasonable to conclude a similar MOA for other effects of iAs, such as cardiovascular effects that begin with toxicity to endothelial cells, macrophages and vascular smooth cells. Whereas in bladder, lung and skin the non-cancer toxicities lead to cell death, regenerative proliferation and cancer, in other organs, non-cancer toxicities have other consequences, depending on the specific organ/tissue involved.. These toxicities may include adverse events that do not necessarily lead to cell death.

In summary, there is a common MOA for cancer and non-cancer effects of inorganic arsenic, with cancer beginning as a non-cancerous lesion, cytotoxicity: exposure to sufficiently high levels of iAs generates adequate concentrations of trivalent arsenicals that react with sulfhydryl groups of critical cellular proteins producing a response (Figure 7). For epithelial tissues, the response is cytotoxicity with cell death leading to consequent regenerative proliferation that increases the risk of cancer (Figure 5), whereas in non-epithelial tissues, the cytotoxicity response leads to other adverse cellular changes and other disease sequelae.

The cytotoxicity and the following effects of arsenic are caused by interaction of the reactive trivalent arsenicals with sulfhydryl groups, and lead to the adverse reactions, whether precancerous or not (Cohen et al., 2006a, 2007; Kitchin Wallace, 2005, 2008). There are differences between species and also between tissues. These differences are related to the availability of sulfhydryl groups in the specific proteins which are affected, and to the affinity of the enzymes of the various animal species to the various metabolites of the various animal species to the metabolites of iAs. Most proteins have relatively short half-lives, therefore, cells can readily compensate for the loss of small amounts of any protein, due to an adequate reserve capacity or rapid replacement with new protein molecules. Thus, only a substantial (threshold) amount of reactive trivalent metabolites will cause an effect. Low concentrations of iAs will lead to adaptive responses that are rapidly and completely compensated for by homeostatic mechanisms that are available to the whole organism (Clewell et al., 2011; Gentry et al., 2010). The adaptive responses activate metabolic processes and transport mechanisms to facilitate inactivation and/or excretion of arsenic. Furthermore, like other chemicals, an adaptive response is consistent with the observation that cytotoxicity does not occur with any of the trivalent or thiolated arsenicals below a certain concentration, either *in vitro* or *in vivo*.

Conclusions

iAs, at high exposures, is a known human carcinogen that targets mainly the urinary bladder, lung and skin. Substantial evidence, from both *in vitro* and *in vivo* investigations, supports a MOA that involves cytotoxicity followed by regenerative proliferation, which, if prolonged, leads to an increased risk of cancer in the urothelium, the bronchial epithelium and the epidermis. The cytotoxicity is produced by reactive trivalent arsenic metabolites that react with sulfhydryl groups in critical cellular proteins. Non-cancer toxicities associated with high iAs exposures likely occur by the same MOA of trivalent arsenicals reacting with sulfhydryl groups of critical cellular proteins, in which cases the cytotoxicity is not followed by prolonged regenerative proliferation, but other toxicological responses ensue.

Cancer induced by a MOA that involves cytotoxicity and regenerative proliferation is an accepted threshold phenomenon for chemicals, and the available evidence supports this MOA for iAs. Epidemiologic studies, old and more recent, in the US and worldwide, consistently support a threshold for iAs-associated cancers at exposure of humans to iAs at concentrations above 100 μg/L. Environmental exposures to iAs in the United States generally do not reach this threshold.

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Declaration of interest

The employment affiliation of the authors is shown on the cover page. Dr. Cohen, Dr. Beck, Ms. Lewis, and Ms. Arnold have received financial support from the Arsenic Science Task Force, managed by B&C Consortia Management, for the preparation of this manuscript. The Arsenic Science Task Force is an organization of companies and trade associations that fund research and analyses to inform ongoing scientific and regulatory assessments of arsenic. Dr. Eldan is employed by Luxembourg Industries LTD, which develops and produces crop protection and specialty chemical products, including organic arsenical products. Dr. Beck has been an expert witness in legal matters pertaining to the toxicology and epidemiology of arsenic, however, she has not relied on this manuscript in presenting her opinions. Dr. Cohen has presented a brief summary of these finding to the U.S. Environmental Protection Agency (National Center for Environmental Assessment and Office of Pesticide Programs), to the U.S. Food and Drug Administration, and at a symposium held by the National Research Council. The responsibility for the preparation and content of this manuscript rests with the authors, and the conclusions and interpretations expressed are entirely those of the authors and not of any institution or commercial entity.

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